School of Land and Food

Reproduction and Fertility Parameters of Dairy Cows Supplemented with Monounsaturated Fatty Acid-rich Canola Oil: mRNA Gene Expression

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Declaration

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John Roger Otto

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Thesis Publications

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2. Otto JR, Malau-Aduli BS, Rao A, Clarke IJ, Malau-Aduli AEO 2014. Effect of incremental levels of crude degummed canola oil on milk progesterone, plasma luteinizing and follicle stimulating hormones of primiparous Holstein-Friesian cows in a pasture-based system. *International Journal of Veterinary Science and Medicine* 2: 122-129, doi:10.1016/j.ijvsm.2014.10.001

3. Otto JR, Malau-Aduli BS, Nichols PD, Malau-Aduli AEO 2014. Influence of supplementing pasture-based primiparous Holstein-Friesian dairy cows with crude degummed canola oil on milk fatty acid composition. *Journal of Nutritional Therapeutics* 3 (2): 55-66, doi: http://dx.doi.org/10.6000/1929-5634.2014.03.02.4

4. Otto JR, Freeman MJ, Malau-Aduli BS, Nichols PD, Lane PA, Malau-Aduli AEO 2014. Reproduction and fertility parameters of dairy cows supplemented with omega-3 fatty acid-rich canola oil. *Annual Research and Review in Biology*, 4 (10): 1611-1636, doi: 10.9734/ARRB/2014/7689

5. Malau-Aduli AEO, Otto JR, Suybeng B, Kashani A, Lane PA, Malau-Aduli BS, Nichols PD, 2015. Effect of supplementation with crude degummed canola oil on the expression of fat-related genes involved in reproduction and lipogenesis in primiparous Holstein-Friesian dairy cows in a pasture-based system. *Adv Genet Eng*, 4: 123, doi: 10.4172/2169-0111.1000123

6. Otto JR, Nish P, Balogun RO, Freeman MJ, Malau-Aduli BS, Lane PA, Malau-Aduli AEO 2015. Effect of dietary supplementation of pasture-based primiparous Holstein-Friesian cows with degummed crude canola oil on body condition score, liveweight, milk yield and composition. *Journal of Applied Animal Research*, 1-7, doi: 10.1080/09712119.2015.1031768

Peer-reviewed /Edited Conference Papers

7. Malau-Aduli AEO, Otto JR 2013. Genetic and environmental variations in reproductive performance of pasture-based dairy cows. *Proceedings of the 11th World Conference on Animal Production*, 15-20th October 2013, Beijing International Convention Centre, Beijing, China, pp.117

8. Malau-Aduli AEO, Otto JR, Nish P 2013. Lactation performance of purebred and crossbred dairy cows on pastures and impact on fertility. *Proceedings of the 11th World Conference on Animal Production*, 15-20th October 2013, Beijing International Convention Centre, Beijing, China, pp.84

Thesis Abstract

The main objective of this thesis was to investigate the effects of supplementing pasture-based, primiparous, Holstein-Friesian cows with incremental levels of crude degummed canola oil (CDCO) on milk production, fatty acid composition, plasma metabolites, hormonal profiles and the expression of fat-related genes involved in reproduction and lipogenesis. It was generally hypothesised that *CDCO supplementation would influence liveweight, body condition score, milk yield and fatty acid composition, plasma metabolites and reproductive hormonal profiles and mRNA expression of fat-related genes under temperate Australian environmental conditions*. A random allocation of cows into treatment groups that consisted of a wheat-based pelleted diet with no supplemental CDCO (control), or with CDCO added at 25 ml/kg (low), 35 ml/kg (medium) and 50 ml/kg (high) was employed in an eight-week feeding trial after two weeks of adjustment. All cows had *ad libitum* access to pasture.

The first experiment investigated the effect of CDCO on body condition score, liveweight, milk yield and composition. It tested the hypothesis that *milk yield, fat and protein contents will increase as level and duration of CDCO supplementation increased, while cow body condition score and liveweight will be suppressed.* Results indicated that the duration of supplementation significantly ($P<0.05$) influenced lactation traits and cows receiving CDCO supplements had greater milk yield at the expense of milk fat and protein without any negative impact on body condition score and average daily gain. These initial results provided empirical evidence that in a dairy setting where pasture is the main feed base, energy spared through CDCO-induced milk fat depression is partitioned towards milk yield.

This finding raised a further research question on the impact of supplementation on energy-related plasma metabolites circulating in the lactating cow. Therefore, the second experiment investigated the effect of CDCO and *ad libitum* grazing on plasma metabolite profiles and

tested the hypothesis that *incremental levels of CDCO supplement will decrease plasma non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB), but increase cholesterol and glucose metabolites*. It was demonstrated that with the exception of BHB, CDCO did not alter plasma metabolite profiles (P>0.05), indicating that primiparous cows grazing high quality pastures during spring had sufficient energy intakes to prevent negative energy balance at 40 days in milk without the need for added oil supplements. However, duration of supplementation (week of lactation) had a significant effect (P<0.05) on the concentrations of NEFA, BHB and glucose, thus raising a further research question on the potential of CDCO in improving milk quality for beneficial health effects through the manipulation of milk fatty acid composition. Therefore, the third experiment tested the hypothesis that *supplementation with CDCO will alter milk fatty acid composition towards increased total monounsaturates (tMUFA) and a decrease in total saturates ((tSFA).* Results of significant (P<0.05) increases in 18:1ω9c, 18:1ω7t, *t*MUFA, *t*MUFA/*t*SFA ratio and a decrease in *t*SFA were observed, thus demonstrating improved milk quality and enhanced beneficial healthy fatty acid profile without any negative impact on the animals or milk taste.

The fourth experiment tested the hypothesis that *incremental levels of CDCO will alter the profiles of progesterone (P4), luteinizing (LH) and follicle stimulating (FSH) hormones*. It was apparent that FSH and P4 profiles were significantly (P<0.05) influenced by duration and levels of supplementation, but not LH. Cows in the high (0.459 ng/ml), medium (0.367 ng/ml) and low (0.251 ng/ml) treatment groups had higher FSH concentrations compared to the control (0.172 ng/ml) cows. The fifth and last experiment investigated the effect of CDCO on the expression of *Arylalkylamine N-acetyltransferase* (*AANAT*), *B-cell translocation gene-2* (*BTG2*) and *Fatty Acid Synthase* (*FASN*) genes involved in reproduction and lipid synthesis. The hypothesis tested was that *post-partum supplementation of primiparous Holstein-Friesian cows with dietary CDCO in a pasture-based system will alter*

the relative mRNA abundance and expression of AANAT, BTG2 and FASN genes associated with lipid metabolism. Both level and duration of supplementation with CDCO were significant sources of variation ($P<0.05$) that influenced *BTG2* expression, while the expressions of *AANAT* and *FASN* genes were unaffected ($P>0.05$). Cows in the high (0.67 fold), medium (0.87 fold) and low (0.56 fold) levels of oil treatments had lower expressions of *BTG2* gene compared to the control (1.0 fold) group of cows. It was concluded that the supplementation of grazing cows with lipid-rich feeds could be utilised as a dietary manipulation tool to down-regulate the expression of *BTG2* gene and its anti-proliferative attributes. The low expression of *BTG2* might be important when the reproductive system of cows is recovering from the effect of gestation and new cell growth is required. The suppression of *FASN* gene expression can be beneficial in sparing energy from milk fat synthesis and re-directing the surplus to non-mammary tissues *in vivo*. However, severe milk fat depression may be economically undesirable to dairy farmers because of its contribution to total milk solids upon which milk prices are based. These findings highlight the important role of supplementary nutrition in altering reproductive and lipogenic gene expression in lactating primiparous cows.

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Abbreviations

AA: Arachidonic acid

AANAT: Arylalkylamine N-acetyltransferase

ADF: Acid detergent fibre

ALA: Alpha-linolenic acid

BCS: Body condition score

BEND: Bovine endometrial cells

BFTS: Bentley Fourier Transform Spectrometer

BHBA: β-Hydroxybutyrate;

bIFN-τ: Bovine interferone-tau

BLAD: Bovine leukocyte adhesion deficiency

BLAST: Basic Local Alignment Search Tool

BTG2: B-cell translocation gene 2

Ca: Calcium

CCK: Cholecystokinin;

CDCO: Crude degummed canola oil

cDNA: Complementary DNA

CL: Corpus luteum

CLA: Conjugated linoleic acid

CP: Crude protein

Cp: Cycle number of crossing point

CSIRO: Commonwealth Scientific and Industrial Research Organization

Ct: Cycle threshold

CV: Coefficient of variation

DHA: Docosahexaenoic acid

DIM: Days in milk

DM: Dry matter

DMI: Dry matter intake

DNA: Deoxyribonucleic acid

DPI: Department of primary industry

E2: Oestrogen

EE: Ether extract

E-FLAX: Extracted flaxseed

ELISA: Enzyme-linked immunosorbent assay

EPA: Eicosapentaenoic acid

E-SUN: Extracted Sunflower

FA: Fatty acids

FADS2: Fatty acid desaturase 2

FAME: Fatty acid methyl esters

FASN: Fatty acid synthase gene

FCM: Fat-corrected milk

FS: Flax Seed

FSH: Follicle stimulating hormone

FT-IR: Fourier Transformed Infrared

GC-MS: Gas chromatographic- mass spectrometer

GH: Growth hormone

GHR: Growth hormone receptor

GLC: Gas-liquid chromatograph

GnRH: Gonadotrophin releasing hormone

3β-HSD: 3β-hydroxysteroid dehydrogenase/Δ5, Δ4 isomerase

IGF-1: Insulin like growth factor-1

LA: Linoleic acid

LC-PUFA: Long-chain polyunsaturated fatty acid

LH: Luteinising hormone

MC: Moisture content

ME: Metabolisable energy

MFD: Milk fat depression

MPC: Milking Point Controller

mRNA: Messenger Ribonucleic Acid

MUFA: Monounsaturated fatty acids

ω-3: Omega-3 PUFA

ω-6: Omega-6

NADPH: Nicotinamide adenine dinucleotide phosphate

NDF: Neutral detergent fibre

NEBAL: Negative energy balance

NEFA: Non-esterified fatty acid

NFC: Non-fibrous carbohydrate

OM: Organic matter

P4: Progesterone

Pg: Picogram (10^{12})

$PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$

PGFM: 13,14-dihydro-15 keto $PGF_{2\alpha}$

PPAI: Postpartum anovulatory interval

PPARs: Peroxisome proliferator-activated receptor

PPIA: Peptidyl-prolyl cis-trans isomerasa

PUFA: Polyunsaturated fatty acid

qPCR: Quantitative polymerase chain reaction

RNA: Ribonucleic acid

SAS: Statistical Analysis Software

SCC: Somatic cell count

SCD1: Stearoyl-CoA desaturase 1

SFA: Saturated fatty acids

SGR: Specific growth rate

SREBP-1: Steroyl response element binding protein 1

StAR: steroidogenic acute regulatory

TAG: Triacylglycerol

TIA: Tasmanian Institute of Agriculture

TMR: Total mixed ration.

***t*MUFA:** Total monounsaturated

***t*PUFA:** Total polyunsaturated fatty acids

***t*SFA:** Total saturated fatty acids

UBC: Ubiquitin C

Δ^{12} and Δ^{15}: (delta) desaturase enzymes

Chapter 1 : General Introduction

Milk producing states in Australia have enjoyed tremendous increases in milk production in recent years with an average cow milk yield of 5000-6000 litres per year (Dairy Australia, 2014), which is made realizable by the contribution of high genetic merit Holstein-Friesian cows constituting over 90% of the national dairy herd. The importance of adequate nutrition for high producing cows cannot be overemphasized. In pasture-based production systems, high milk production predisposes cows to severe negative energy balance (NEBAL) because the energy intake from grass alone is mostly insufficient to sustain high milk yield (Stockdale, 2001; Hutchinson *et al*., 2012). Negative energy balance causes a depletion of energy reserves and subsequent adipose tissue remobilisation to support peak lactation with deleterious consequences on reproductive performance (Adewuyi *et al*., 2005). Severe NEBAL has been associated with atypical hormonal profiles, poor follicular development, delayed post-partum cyclicity and poor oocyte and embryo quality, resulting in decreased pregnancy rates and longer calving intervals (Lucy, 2000; Lucy & Crooker, 2001).

Primiparous cows are first-time calvers, hence are generally small framed (85-90 % of mature cow size), still growing, expected to regain post-partum weight loss and continue to produce milk immediately after parturition. The smaller body size makes cows direct targets for bullying by more mature cows and pushes them further down the bottom of the social hierarchy. Consequently, such cows are always the last to be milked and by implication, arrive last in the paddock, thus potentially reducing grazing time. Therefore, primiparous cows tend to suffer severe NEBAL than mature animals in the herd, causing them to have diminished milk production and reproductive performances (Moran & McLean, 2001).

Reproductive success in dairy cows is assessed by normal and regular calving intervals, number of services per conception, days open and rates of conception to first service (Buckley *et al*., 2000; Snijders *et al*., 2001; Olori *et al*., 2002). Calving interval is important because it impacts on herd replacement and farm economic returns (Van Arendonk *et al*., 1989; Esselmont *et al*., 2001). However, calving intervals, number of days open and intervals to first breeding are becoming longer and pregnancy rate to first service declining in diverse dairy systems around the world (Butler 2003, Rocha *et al*., 2010, Malau-Aduli & Otto, 2013) mainly due to NEBAL, atypical reproductive hormonal profiles and decades of dairy cow breeding strategy that emphasised selection for high milk yield at the detriment of reproductive performance (Staples *et al*., 1998).

One strategy to alleviate extreme NEBAL is to utilise high energy-dense supplements like canola. Canola (*Brassica napus. L*) is the third largest important crop grown in Australia after wheat and barley (Kirkegaard *et al*., 1994; Seymour *et al*., 2012). Canola is grown as a cash and break crop in regions with an annual rainfall greater than 450 mm (Cowling, 2007). The area sown to canola crop has risen from 150,000 ha in 1991 to 1.85 million ha in 2011 (Colton & Potter, 1999; Seberry *et al*., 2012). Canola is a product of many decades of genetic engineering with emphasis on the reduction of erucic acid (Stefansson & Kondra, 1975) and glucosinolates in the parent rapeseed (Sharma *et al*., 1977; Lardy & Kerley, 1994; Hristov *et al*., 2011). Conventional canola oil contains high concentrations of polyunsaturated fatty acids (PUFA) in comparison to the parentage line of rapeseed (Hristov *et al*., 2011). Although good in production of more PUFA, canola oil when subjected to frying heat becomes rancid and produces more *trans*-FA, a major cause of cardiovascular diseases (Mensink *et al*., 2003). As a consequence, the modern canola plant is capable of producing oil with a greater concentration of ω-6 and adequate ω-3 PUFA (Sakhno, 2010).

In response to health concerns, research interests in modifying milk FA composition toward less saturated medium-chain FA and more LC-PUFA are on the increase. The simplest way of altering milk fat composition is to supplement the diets of cows with unsaturated lipids (Hristov *et al*., 2011). Several studies have been published on the impact of dietary lipid supplements on milk fat composition (Glasser *et al*., 2008). Although different proportions and relative abundance of shorter chain and long chain polyunsaturated fatty acids (LC-PUFA) in canola oil had been reported or shown to modify milk quality (Chichlowski *et al*., 2005), and at the same time, alter metabolite and hormonal profiles, and gene expression patterns in dairy cows (Angulo *et al*., 2012), studies investigating the impact of dietary fat supplementation (particularly canola oil) on milk fatty acid profiles in pasture-based primiparous cows are lacking. Therefore, information is required about the impact of supplementing lactating cows with CDCO on milk fatty acid composition.

The energy status of a cow is reflected by the proportions of circulating non-esterified fatty acids (NEFA) and beta-hydroxy butyrate (BHB) in the blood (Grummer & Carroll, 1991; Leroy *et al*., 2005; Colazo *et al*., 2009; Lopes *et al*., 2011). The effect of dietary fat supplements on plasma metabolites in dairy cows has been inconsistent and highly variable in the published literature (Khorasani & Kennelly, 1998). Previous research finding suggests that dietary supplementation with fat sources containing adequate proportions of unsaturated fats could potentially improve fertility in high merit dairy cows (Santos *et al*., 2008) through regulation of the expression of fat related genes (Perez *et al*., 2010; Jeckel *et al*., 2014) and *de novo* fat synthesis (Hutchinson *et al*., 2011). Understanding the molecular mechanism underpinning the impact of dietary fat intake on dairy fertility sequences from oestrous to conception could revolutionise how nutrition is managed in dairy farms to improve reproduction performances. It will also assist researchers in unravelling the intricate biological mechanisms involved with feeding dietary fats to grazing cows and their effects on

lactation and fertility traits. This will enable dairy farmers make informed choices and tailored decisions when feeding lactating cows with specific dietary fat supplements utilising an effective and long-term nutritional strategy that can assist in a better understanding of nutrition-fertility interactions as a potential solution to the sub-fertility problem in dairy cows.

Therefore, the overarching objective of this thesis was to investigate the effect of incremental levels of CDCO on liveweight, milk yield, FA composition, plasma metabolites, hormonal profiles and relative mRNA expression of fat related genes involved in reproductive and lipogenesis in primiparous pasture-based Holstein-Friesian dairy cows. The thesis is structured into the following chapters:

Chapter 1: General Introduction

Chapter 2: Literature Review: The literature review is an in-depth exploration of published literature on reproductive performance in cows, the effect of body condition score and NEBAL on fertility parameters, and the impact of dietary fat supplementation on lactation, milk composition and liveweight traits in diverse dairy systems around the world.

The successive chapters are investigative experimental studies that describe the effect of dietary fat supplementation on milk FA composition, plasma metabolites, hormonal profiles and mRNA expression of reproductive and lipogenic genes.

Chapter 3: The main objective of this chapter was to investigate the effect of CDCO on lactation performance, milk composition and liveweight traits. The tested hypothesis was that *supplementing primiparous Holstein-Friesian cows in a pasture-based dairy system with CDCO will increase milk yield, fat and protein contents, but decrease cow BCS and liveweight traits.*

Chapter 4: The objective of the study was to quantify milk fatty acid composition of cows supplemented with CDCO with a view to improving the milk quality for beneficial health effects. In this chapter, it was hypothesized that *incremental supplementation of grazing primiparous Holstein-Friesian cows with CDCO will alter milk fatty acid composition towards increased total monounsaturates.*

Chapter 5: The objective was to investigate changes in plasma metabolite profiles. The hypothesis tested in this study was that *incremental levels of CDCO supplement will decrease plasma NEFA and BHB, but increase plasma cholesterol and glucose.*

Chapter 6: This study's objective was to determine whether dietary inclusion of CDCO at incremental levels for eight weeks will have significant influence on the concentrations of P4, LH and FSH in primiparous Holstein-Friesian cows grazing pastures. The hypothesis tested was that *postpartum supplementation with CDCO will alter the concentrations of progesterone (P4), luteinizing hormone (LH) and follicle stimulating hormone (FSH).*

Chapter 7: The objective of this study was to investigate the effects of CDCO on messenger Ribonucleic Acid (mRNA) expression of Aralkylamine N-acetyltransferase (AANAT), B-cell translocation gene-2 (BTG2) and Fatty Acid Synthase (FASN) genes involved in reproduction and production. The hypothesis tested was that *postpartum supplementation with CDCO will alter the expression of AANAT, BTG2 and FASN.*

Chapter 8: This chapter is a general discussion and conclusion of the main results of the study and areas warranting further investigations.

Appendices: Contains all supplementary materials and copies of peer-reviewed publications from the thesis.

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Chapter 2 : Literature Review

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Abstract

Dietary supplementation of lactating dairy cows with fat has long been used as a management tool to increase the energy density of feeds for improving milk production, reproduction and alleviating the menace of postpartum NEBAL. In this chapter, we show that attempts had been made to investigate the effects of ω-3 PUFA on reproduction and fertility parameters but the results have been diverse and inconsistent. The effects of ω-3 PUFA on prostaglandin F2α (PGF2α) synthesis are well established, however, little is known about the effect of ω-3 PUFA on P4, oestrogen (E2), LH, FSH, insulin-like growth factor-1(IGF-1) and fat related genes linked to reproductive performance. There is contrasting evidence linking ω-3 PUFA with ovulation, oestrous cycle, PGF2α and P4 production. In addition, the mechanisms behind the influences of ω-3 PUFA on reproductive traits such as number of services per conception and embryo survival are not well understood. Therefore, this paper reviews the effect of ω-3 PUFA on cow reproduction and the causal factors of fertility problems in the dairy industry. It also substantiates the need to establish a better understanding of the interactions between ω-3 PUFA and cow fertility, with the aim of ameliorating the progressive subfertility issues emerging in the dairy industry. This review also summarises the identified knowledge gaps and highlights fruitful directions for future research in high producing dairy cows in pasture-based systems.

Keywords: Polyunsaturated fatty acid; prostaglandin F2α; progesterone; reproductive traits; fat supplementation.

Introduction

The high-yielding, modern dairy cow is a product of many decades of genetic selection that continually laid emphasis on milk yield through the utilization of progeny tested bulls, sexed semen technology, improved management technologies and better nutrition (Rodriguez-Martinez *et al*., 2008). However, the genetic progress resulting in increased milk production has led to a gradual but progressive decline in reproductive performance in diverse dairy production systems throughout the world (Royal *et al*., 2000; Royal *et al*., 2002; Butler, 2003). The lengthening of calving intervals in dairy cattle has already been observed in the USA, United Kingdom and Portugal; where an annual decline of 1.7 days in inter-calving interval was reported by Rocha et al. (2010). Studies by Royal et al. (2000) and Butler (2003) also confirmed a decline in pregnancy rates from 55.6% to 39.7% at a potential rate nearing 1% annually.

Dairy farmers located in the southern region of Australia are largely dependent on pasture as the main feed source, neglecting supplementation with grains because of the associated high price (Adediran *et al*., 2010). Stockdale (2001) outlined the fact that most Australian dairy farmers are now heavily reliant on the Holstein-Friesian breed from North America. The contribution of the Holstein-Friesian to the Australian dairy industry is very high. However, to continuously achieve high milk production, it is essential that Holstein-Friesian cows are fed energy-dense feeds (Stockdale, 2001; Schroeder *et al*., 2004; Dairy Australia, 2013). Generally, pasture alone is insufficient to meet the energy requirements of high merit dairy cows particularly during the postpartum period. During the postpartum phase, the lactating cow experiences an increase in both physiological and metabolic processes resulting in escalated nutritional demands to maintain continuous milk flow (Roche *et al*., 2009). In the USA, most dairy farmers use total mixed rations (TMR) to provide adequate nutrition for high yielding cows (Nocek *et al*., 1986), whereas Australian dairy farmers are still mostly

reliant on pastures (Stockdale, 2001). The pasture-based dairy system as practiced in Australia, attempts to obtain uniform annual calving patterns within which parturition is rigorous during early to late spring to allow thorough consumption of the lush spring pasture growth (Stockdale, 2001). However, to achieve high milk yields over the lactation period and maintain a yearly calving interval, it is paramount that dairy cows resume cyclicity, ovulate, conceive early postpartum and carry the pregnancy to term (Rhodes *et al*., 2003).

A typical dairy cow experiences NEBAL at the beginning of lactation. This is because the energy requirements for both metabolic processes and milk synthesis outweigh the amount of energy being replenished through dry matter intake (DMI; Butler, 2000; Lucy & Crooker, 2001; Chagas *et al*., 2007). Some authors have further proposed that the nadir DMI at early lactation may be related to a linear increase of plasma cholecystokinin (CCK; Opara *et al*., 1994; Choi & Palmquist, 1996; Benson *et al*., 2001). Plasma CCK is a pancreatic polypeptide hormone that regulates satiety and feed intake in animals (Benson *et al*., 2001). Grovum (1981), McLaughlin and Baile (1981), and Faverdin (1986) found that intravenous injection of CCK in cow, sheep and mice resulted in reduced appetites. Bauman and Bruce-Currie (1980) found that NEBAL of lactating cows usually continued until 6-8 weeks postpartum. In an attempt to sustain a continuous flow of energy and maintain copious milk synthesis postpartum, the liver elevates the rate of gluconeogenesis in the body. When plasma glucose levels decrease, body fat remobilisation is instigated from nutrient accrual to provide sufficient energy that can maintain continuous milk production until the animal returns to a positive energy status. Grummer (1995), Bauman and Griinari (2001), and Adewuyi et al. (2005) found that cows suffering from NEBAL have increased concentrations of serum glucagon and growth hormone (GH), whereas the concentrations of insulin and IGF-I are decreased, indicating that NEBAL is heavily influenced by high milk production. Therefore, it is important that cows receive adequate nutrition prepartum and postpartum to meet their

energy needs in order to minimize the production of NEFA which could potentially compromise the reproductive performance of dairy cows. The onset of parturition is usually accompanied by high energy demand to support high milk production, however in the period leading up to the transition period (2-3 weeks prior to parturition), it is usual for the DMI of high merit cows to be at nadir and therefore the energy requirement for lactating cows is compensated through remobilisation of adipose tissue by lipolytic process. Lipolysis of adipose tissues leads to a surge of fatty acids that are reversibly bound to albumin in the plasma (Adewuyi *et al.*, 2005). Plasma NEFA has been associated with poor follicle and granulosa cell development (Beam & Butler, 1999; Vanholder *et al*., 2005).

One suggested nutritional method for improving the energy and fertility status of lactating cows both prepartum and postpartum, is the addition of fat supplements to the cow's diet (Santos *et al*., 2008; Hutchinson *et al*., 2012). De Veth et al. (2009) conducted five controlled meta-analysis studies and found that dairy cows supplemented with conjugated linoleic acid (CLA) decreased the median time to first ovulation. A study in Wisconsin showed that cows fed with supplements containing long-chain polyunsaturated fatty acid (LC-PUFA: ≥20) exhibited stronger oestrus, had more active ovaries and less exogenous PGF2α was required for oestrous induction (Scott *et al*., 1995). Other studies have also demonstrated that supplementation of dairy cows with fat was consistent with an increased concentration of cholesterol (Grummer & Carroll, 1991; Ball & Peters, 2004) and arachidonic acid in the follicular fluids (Elliot & Elliot, 2005). Cholesterol is reported to be the precursor for the synthesis of steroid hormones, P4 and E2, while arachidonic acid is the precursor for PGF2α (Staples *et al.*, 1998; Ball & Peters, 2004; Santos *et al*., 2008). E2, PGF2α and P4 are key hormones involved in ovulation, oestrous cycle and maintenance of pregnancy in dairy cows (Campbell *et al.*, 2003; Ball & Peters, 2004; Field & Taylor, 2008).

To our knowledge, the response of pasture-based dairy cows to supplementation with canola oil containing ω-3 PUFA and the subsequent impact on reproduction and fertility parameters is not well known. Examination of literature reveals that fat supplementation trials in dairy cows have been mainly nutritional in focus and inconsistent, without deliberate evaluation of the impact on reproductive and fertility parameters. The mechanisms by which LC-PUFA affects fertility in dairy cows are also largely unknown. However, as suggested by Lucy et al. (1991) and Staples et al. (1998), some mechanisms by which fat could influence reproductive performance include: follicular growth through insulin manipulation, inhibition of PGF2α affecting longevity of the corpus luteum (CL) and overall improvement in the energy status of cows. The main objective of this chapter is to review the studies conducted on effects of fat supplementation on reproductive parameters in bovines. This paper also aims to explore the proposed mechanisms of fat metabolism and impacts on vital reproductive hormones and plasma metabolites. It also reviews the causes of reproductive problems in the dairy industry, summarises the identified knowledge gaps and highlights fruitful directions for research aimed at unravelling the specific effect of ω-3 PUFA on reproduction and fertility parameters of high-producing dairy cows.

Findings

Canola Oil

Canola plant (*Brassica napus L*.) is a product of many decades of genetic engineering with emphasis on the reduction of erucic acid (Stefansson & Kondra, 1975) and glucosinolates present in the parent rapeseed (Sharma *et al.,* 1977; Lardy & Kerley, 1994; Hristov *et al*., 2011). Conventional canola oil contains high concentrations of PUFA in comparison to the parentage line of rapeseed (Hristov *et al*., 2011). Although good in production of more PUFA, canola oil when subjected to frying heat becomes rancid and produces more *trans*-fatty acids, a major cause of cardiovascular diseases (Mensink *et al*., 2003). As a consequence, the modern canola plant is capable of producing oil with greater concentrations of ω-6 and adequate ω-3 PUFA (Sakhno, 2010). The different proportions and relative abundance of shorter and LC-PUFA in canola oil have been implicated in alteration of fatty acid profiles in the plasma and milk fat of dairy cows (Chichlowski *et al*., 2005).

Fat supplementation and substitution effect

Supplementation of grazing cows with lipid/fat sources causes a reduction on DMI, an effect termed substitution rate (Kellaway & Porta, 1993). Substitution rate is the measure of pasture DMI in unsupplemented treatment minus pasture DMI in supplemented treatment divided by supplement DMI (Bargo et al., 2003). It has been reported that substitution rate can range from 0-1.2 kg pasture/kg concentrate fed to cows consuming fresh lush pasture (Kellaway & Harrington, 2004; Bargo et al., 2003; Hulme et al., 1986; Cowan et al., 1977). Substitution rate is known to have the greatest effect in high pasture allowance dairy system, particularly in cows supplemented with fine, energy dense supplements (Stockdale, 1999). Conversely, protein supplements produce lower substitution effects than finely processed grains and fat supplements (Kellaway & Harrington, 2004). Dairy cows consuming finely processed

supplements have rapid digestion; this in effect lowers rumen pH (Dixon and Stockdale, 1999). Consequently, a lowered rumen pH causes the death of cellulolytic rumen bacteria and this causes reduction in fibre digestion and affects pasture DMI (Bargo et al., 2003). Buffers can be used to neutralise rumen pH to an acceptable level (Kellaway & Harrington, 2004). Factors affecting substitution rate can be divided into pasture, supplement and animal related factors. The pasture related factors include pasture allowance, height, species, mass, and quality. Supplement factors include amount, type, chemical and physical properties, while the animal factors include genetic merit, production level and stage of lactation (Bargo et al., 2003).

Pasture allowance

The DMI of a dairy cow is positively related to the amount of pasture provided, mainly because as pasture allowances increases, the ease of pasture harvest by a cow also increases (Stockdale et al., 1997). Substitution rate is primarily affected by the amount of pasture intake when supplements are included in the ration (Kellaway & Harrington, 2004). Previous experiments investigating the influence of pasture allowance on substitution rate reported that at the rates of 17.1 and 33.2 kg DM/cow/day of pasture, substitution rates of 0.25 and 0.69 were observed (Grainger & Mathews, 1989). Feeding dairy cows with pelleted concentrates at the rate of 4 kg/cow/day and pasture at 15 kg/cow/day and 45 kg/cow/day had substitution rates of 0.02 and 0.3 kg/kg, respectively (Robinson & Rogers, 1983). A study with high producing cows fed 7 kg DM/day of concentrate and allowed pasture at 25 kg/cow/day and greater than 25 kg/cow/day, had substitution rates of 0.20 and 0.62 kg pasture/kg concentrate, respectively (Bargo et al., 2002). These previous studies indicate that a significant linear relationship exists between pasture allowance and substitution rates refer to Table 2.1. Therefore, substitution rate in cows supplemented with dietary fat sources in pasture-based system has an impact on production and reproductive performances.

Table 2.1 Substitution rates at various pasture allowances and levels of supplementation

Reference	Pasture type	Pasture allowance (kg/day)	Supplement type	Amount of supplement (kg/day)	Substitution rate (kg/kg)	Milk yield response (kg/kg supplement)
Grainger and Mathews, 1989	Ryegrass and clover	7.6	Grain-based pellet	3.2	0.00	0.97
		17.1		3.2	0.25	0.69
		33.1		3.2	0.69	0.28
Robinson and Rogers, 1983	Temperate pasture	15.0	Grain-based pellet	4.0	0.02	0.50
		45.0		4.0	0.30	0.02
Stockdale and Triggs, 1985	Predominantly paspalum	15.0	Pellets	2.0	0.00	1.60
				4.0	0.00	0.80
				Ad libitum	0.23	0.70
				2.0	0.94	1.20
		26.0		4.0	0.43	0.80
				Ad libitum	0.30	0.50
Opatpatanakit et al., 1992	Ryegrass and clover	48.2	Rolled barley	4.0	0.64	0.10
		47.1		8.0	0.63	0.10
Robaina et al., 1998	Ryegrass and clover year 1,	18.0	Barley and lupin	4.4	1.14	0.73
		35.0		4.4	0.98	0.66
	Ryegrass and clover year 2	21.0	Barley and lupin	4.2	0.21	1.13
		42.0		4.4	0.45	0.80
Wales et al., 2001	Ryegrass and clover	19.0	Grain-based pellet	5.0	0.18	1.00

Source: Kellaway and Harrington (2004)

Lactation stage

During early lactation, the energy demands for milk production and maintenance are very high. As a result, adipose tissue remobilisation is triggered to support milk yield (Kellaway & Porta, 1993). During late lactation, attention is more focussed toward liveweight gain and maintenance to prepare for reproduction, and therefore, energy is partitioned toward body tissue repair (Kellaway & Porta, 1993). Therefore, the effect of substitution rate tends to be lower in late lactating cows than during early lactation. This is supported by previous studies which found that substitution rate declines as lactation progresses (Phipps et al., 1987). An experiment by Stockdale et al. (1987) reported that the greatest response in milk yield to varying levels of supplementation was greatest in early lactation than in late lactation.

However, some studies also found that consumption of poor quality pasture by dairy cows in late lactation leads to a greater response in milk yield to supplementation than cows grazing good quality pasture in early lactation (Kellaway & Harrington, 2004).

Type and chemical properties of supplement

Substitution rate occurs differently depending on the type, physical and chemical properties of supplements provided to dairy cows (Bargo et al., 2003). Finely ground pelleted supplements along with grain concentrates are known to have high substitution rates in grazing cows, whereas protein supplements have low substitution rates (Kellaway & Harrington, 2004). The substitution rates for barley and cotton meals were reported to be 0.64 and 0.39, respectively (Paynter & Rogers, 1982). The low substitution rate with protein supplements is due to their ability to degrade slowly in the rumen, than finely ground pelleted feeds which degrade rapidly and lower rumen pH (Kellaway & Harrington, 2004). A study by Valentine & Bartsch (1987) found that the rumen pH of cows consuming protein diets was maintained above 6.0, while pH in high energy diets decreased to 5.4. Energy dense supplements affect rumen fermentation by disrupting and lowering the digestibility of other diets. Protein supplements increase rumen digestibility by providing extra amino acids and peptides that increase the efficiency of microbial protein synthesis and improve DMI (Roffler et al., 1982; Maeng et al., 1976). The proposed mechanism by which fat added to diets affects pasture intake and rumen fermentation is due to coating effect on rumen pH, antimicrobial properties and modification of cellulose degrading microbes in the rumen (Jenkins, 1993). Lipids in the rumen are known to cover the feed particles to prevent direct attachment of microbes to feed particles which in turn, enhances cellulose digestion in the rumen (Cheng et al., 1991). Fatty acids are able to attach themselves to the microbial membranes because of their hydrophobic and amphiphilic nature (Jenkins, 1993). This antimicrobial nature of lipids

affects cellulolytic microbial population and cellulose digestion (Luvisetto et al., 1987; Borst et al., 1962).

Essential fatty acids in pasture

In dairying regions of Australia, pasture is the main source of lipids in a cows' diet. Essential fatty acids in pasture include ALA (18:3), LA (18:2) and palmitic (16:0) FA. The concentrations of these fatty acids tend to vary with grass species, cultivars and management practices (Mayland et al. 1976; Dewhurst et al. 2003). The concentrations of ALA and LA decrease with increasing intensity of light, temperature and physiological maturity of pastures (Boufaïed et al., 2003; Dewhurst et al., 2001). Correct management protocols that increase vegetation growth increase the levels of FA in pastures (Barta 1975). The application of nitrogen fertiliser was reported to increase the concentrations of all FA in pasture (Elgersma et al., 2005). A study conducted in Ireland found that intensive management practices that prevent early flowering of pasture increase the concentration of LA (Dewhurst et al., 2002). Environmental factors such as shading and wilting are known to reduce the concentration of LA in pastures (Dewhurst & King, 1998). The outcomes of these studies suggest that the concentration of FA in pasture depends on the ratio of stem/ leaf.

Fresh and well-maintained pastures in Australia generally contain between 2-3% fat, with the largest proportion of fatty acid being LA (Elgersma et al., 2003; Dewhurst et al., 2001). Poor management of pastures negatively affects the concentration of LA. Since the lipid content in pastures is very low (2-3%), the amount of post-rumen essential FA is negligible due to the extensive hydrolysis and biohydrogenation in the rumen. This premise is supported by the work of White et al. (2001) who found that the fatty acid content in milk of cows consuming pastures were constant, whereas cows confined and consuming total mixed rations had increased CLA content. However, the addition of dietary fat to the ration of dairy cows causes a substitution effect, especially in pasture-based dairy systems. It has been suggested

that fat supplements containing unsaturated fatty acids increase the amount of post-rumen long chain fatty acids to affect changes in vivo (Drackley, 1999).

Fatty Acid Metabolism

Plant and animal materials contain organic complexes that can readily be dissolved in organic solvents. These complexes are referred to as lipids (Maynard *et al*., 1979; McDonald *et al.,* 1988), as depicted in Table 2.2. Fat is by far the most significant lipid that is of nutritional, metabolic and physiological importance to animals (Maynard *et al*., 1979). The physical compartments of most animal cells rely heavily on fat-generated energy to enable the cells to work and function adequately (Mattos *et al.,* 2000; Santos *et al*., 2008). Fat is simply defined as an ester of FA with glycerol (McDonald *et al*., 2011). Esterification of the trihydric alcohol glycerol by FA results in compounds known as triacylglycerols (TAG; D'Mello, 2000). Structurally, they generally have unbranched carbon chains and a single carboxyl group (Elliot & Elliot, 2005). Fatty acids occur in either saturated or unsaturated forms (Maynard *et al*., 1979).

Table 2.2 Types and classification of lipids

Lipids		
Glycerol based		Non-glycerol based
Simple	Complex	Waxes Steroids
Fats	Glycolipids	Terpenes
	Phospholipids	Eicosanoids

Source: Maynard et al. (1979) and McDonald et al. (1988)

Sources of omega-3 (ω-3) and omega-6 (ω-6) PUFA

The ω-3 and ω-6 LC-PUFA are primarily the fatty acids of interest in dairy reproduction studies (Gulliver *et al*., 2012). The major sources of ω-3 and ω-6 PUFA are fish, vegetables and plant oil (Mattos *et al.*, 2000; Santos *et al*., 2008). The LC-PUFA eicosapentaenoic acid (EPA, 20: 5ω-3) and docosahexaenoic acid (DHA, 22: 6ω-3), are mainly found in fish. However, microalgae are also a major source, while the SC-PUFA such as α-linolenic acid (ALA) and linoleic acid (LA) largely originate from plants (Lands, 1992; Alhazzaa *et al*., 2011; Gulliver *et al*., 2012). Most of the ω-3 PUFA found in dairy cow diets are obtained from grazing pasture (Dewhurst *et al*., 2006). Plants and vegetables with prominent levels of LA, oleic acid and ALA include; sunflower, rapeseed, flaxseed, corn, safflower, linseed, soybean, echium, peanuts and canola (Chichlowski *et al*., 2005; Chong *et al.*, 2006; Miller *et al.*, 2008). Previous studies have reported improved reproductive performance of dairy cows with fat/oil supplementation.

Structure of PUFA

The primary characteristics of the ω-3 PUFA structure are the numbers and positions of the double bonds, chain length and the types of isomers formed (Mattos *et al.*, 2000), as shown in

Figure 2.1. The position of the first double bond relative to the terminal methyl group of the fatty acid is essential for grouping PUFA, e.g. EPA contains 20 carbon atoms, with five double bonds, with the first double bond at the third carbon from the methyl end, thus making it an ω-3 LC-PUFA (EPA, 20:5ω-3; Holub & Holub, 2004). Enzymes such as *desaturase* and *elongase* are responsible for changing the structure of fatty acids (Cook & McMaster, 2002). Consequently, the changes in the carbon chain length of the FA and the position of the double bond influence the biochemical properties and functions of unsaturated FA (Elliot & Elliot, 2005). These structural and chemical changes affect cattle reproduction (Staples *et al.*, 1998; Mattos *et al.*, 2000).

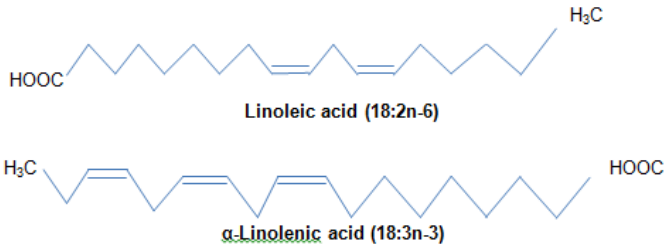

Figure 2.1 Structures of linoleic and alpha-linolenic fatty acids

Source: (Gulliver et al., 2012)

Fat metabolism in the rumen

Metabolism is the term used to illustrate the sequence of chemical processes taking place within living organisms. Metabolism is divided into two parts; catabolism and anabolism (McDonald *et al.*, 2011). Metabolism of lipids in the rumen is facilitated by the rumen indwelling microorganisms, in particular, bacteria and protozoa (Harfoot & Hazlewood, 1997). Bacterial lipase hydrolyses TAG and phospholipids consumed in food (McDonald *et al.*, 1995). Once the FAs are liberated from their ester linkages, the end products (glycerol and NEFA) are utilised in the biohydrogenation process (D'Mello, 2000).

Rumen biohydrogenation and fatty acid synthesis

Biohydrogenation is an extensive microbial process that involves the addition of hydrogen molecule to unsaturated free fatty acids concentrated in the rumen (Doreau & Chilliard, 1997). During biohydrogenation, unsaturated FA (LA and ALA) are extensively hydrogenated to form saturated FA (stearic acid 18:0 and palmitic acid 16:0; D'Mello, 2000). The process of biohydrogenating linoleic acid to stearic acid is demonstrated in Figure 2.2.

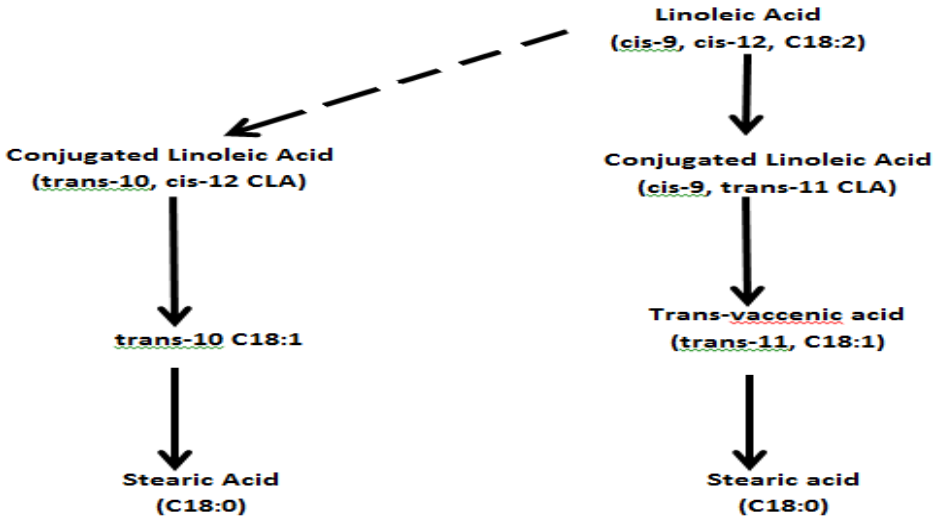

Figure 2.2 Pathways for rumen biohydrogenation of linoleic to stearic acid by microbes

Source: Bauman and Griinari (2001) and D'Mello (2000)

Following biohydrogenation, the saturated and unsaturated FAs that escape this process are subsequently absorbed in the small intestine. As a result of rumen biohydrogenation, approximately 85% and 15% saturated and free FA, respectively, are transported into the small intestine and this process illustrates the efficiency of rumen microbes (Doreau & Chilliard, 1997; D'Mello, 2000). Rumen biohydrogenation is the major factor affecting the delivery of LC- PUFA in the small intestine and subsequent transportation in the blood of ruminants (Wachira *et al*., 2000; Santos *et al*., 2008). Attempts have been made to produce rumen protected lipids (Giesy *et al*., 2002). Currently, the feeding of fats as calcium (Ca)

salts is the main method used to protect fat from rumen biohydrogenation (Giesy *et al*., 2002; Perfield II *et al*., 2002).

Feeding System and Reproductive Performance

Australian southern states are known for their dairy systems that are largely reliant upon pasture as the main feed source where calving is managed to coincide with spring pasture growth peaks. The objective of calving in spring is to allow dairy cows to take full advantage of the abundant lush pasture available during this period to increase milk production (Adediran *et al*., 2010), while at the same time, bolstering early return to cyclicity (Cummins *et al*., 2012). Although the pasture system is cost effective, it cannot maintain the required BCS postpartum (Stockdale, 2000, 2001).

Body condition score (BCS)

In his review, Stockdale (2001) defines BCS as the subjective measure of subcutaneous fat tissues, body fat and inter-muscular fat taken at the 12^{th} rib or the rump of a bovine. Different countries use different scales for assessing BCS (Pryce *et al.*, 2001; Adediran *et al*., 2010). For instance, Australia has adopted the 8 point scale, while USA uses the 5 point scale (Wildman *et al*., 1982; Edmonson *et al*., 1989).

The drastic prepartum and postpartum physiological changes in dairy cows utilise large amounts of metabolised energy (Stockdale, 2000). Energy is utilised for milk synthesis and maintaining the body in good condition (Von Soosten *et al*., 2012). Unfortunately, the energy required to sustain milk production postpartum is far greater than that obtainable from the potential feed intake of the cow (Malau-Aduli & Abubakar, 1992; Roche *et al*., 2009). As a consequence, there is continuous remobilisation of fat from peripheral tissue, aided by lipolysis, to enhance the provision of adequate energy for milk production (Sumner & McNamara, 2007). As a result, BCS of the dairy cow drops to nadir postpartum (Butler &

Smith, 1989; Chagas *et al*., 2007; Stockdale, 2001). Research evidence suggests that remobilisation of fat from the adipose tissue can support the synthesis of approximately 7kg of milk per day (Gibb *et al*., 1992). In their review, Chagas et al. (2007) outlined that fatty cows were more at risk of fat remobilisation than lean cows during early lactation. This supports the findings of Garnsworthy and Wiseman (2007), who established a strong association (r^2=0.82) between BCS at calving and during early lactation.

The relationship between BCS and reproductive performance is well documented (Veerkamp *et al*., 2001; Royal *et al*., 2002; Malau-Aduli *et al*., 2004a), however, the results are conflicting. Studies by Veerkamp et al. (2001) and Royal et al. (2002) found a negative correlation between BCS and dairy fertility, while Berry et al. (2003) found a positive relationship, and further indicated that cows with greater genetic excellence for BCS require less service per conception and can also maintain more pregnancies. However, the studies by Royal et al. (2002) and Veerkamp et al. (2001) contained only small datasets with few observations. Dechow et al. (2002) observed longer calving intervals to first service in cows rapidly losing BCS during early lactation as a result of genetic selection for milk production. Conception rate decreases in dairy cows with low BCS at the start of mating (Stockdale, 2001). However, there are inconsistencies in previous reports. For instance, Grainger et al. (1982) found that anoestrous was reduced by 5.7 days in each cow gaining an additional condition score postpartum, whereas Garnsworthy and Jones (1987) observed no effect of differing BCS on days to resumption of oestrus cycle and number of services per conception in cows at parturition. Therefore, managing body reserves prepartum and postpartum through consistent and accurate measurement of BCS could be essential for enhancing reproductive performance in dairy cows.

Supplementation of dairy cows with fat has been shown by some researchers to reduce fat remobilisation during early lactation (Baumgard *et al*., 2000; Castaneda-Gutierrez *et al*., 2005;

Odens *et al*., 2007; Thatcher *et al*., 2010). Fat supplements containing large proportions of ω-3 PUFA have been shown to decrease adipose tissue remobilisation through inhibition of *de novo* mammary milk fat production (Mattos *et al.*, 2000). Decrease in BCS (on average 2.6) during early lactation is a reflection of NEBAL in a dairy cow (Dillon *et al*., 2006). Cows in NEBAL state are prone to greater levels of NEFAs (greater than 0.7 mM) in plasma (Leroy *et al*., 2005; Rukkwamsuk, 2010), which promote increased production of glucagon (Suriyasathaporn *et al*., 2000) and growth hormones (Liesman *et al*., 1995). This increases plasma glucose, an essential metabolite necessary for milk production in dairy cows postpartum (Snijders *et al*., 2001; Kennedy *et al*., 2003).

Research has shown that concentration of plasma growth hormone is greater in high milk yielders compared to low yielders (Hart *et al*., 1978). Lucy and Crooker (2001) detected surges in the concentration of growth hormones prior to calving in high merit cows, signifying that they played a vital role in the initiation of adipose tissue remobilisation for the support of milk production. The physiological role of growth hormones is only realised when they bind to growth hormone receptor (GHR) on target cells, primarily found in the liver and adipose tissue (Roche *et al*., 2009). The production of IGF-1 by the liver regulates the production of GH (Dillon *et al*., 2006) and is triggered when a cow is in a positive energy status (McGuire *et al*., 1995). Growth hormone binds to the growth hormone receptor-1A (GHR-1A) in the liver when the cow is in a positive energy status (Dillon *et al*., 2006). Consequently, the production of IGF-1 increases, resulting in a reduction of growth hormone production via negative feedback signal to the pituitary gland (Dillon *et al*., 2006).

Non-esterified fatty acid

During NEBAL, various hormones modify peripheral tissue reactions to diminish the rate of hepatic lipogenesis and increase the rate of hepatic lipolysis. This in turn optimises the

plasma concentration of NEFA (Spicer *et al.,* 1990; Lucy *et al*., 1992). This is a homeostatic process which results in the provision of energy to the mammary glands for milk synthesis (Adewuyi *et al.,* 2005). The energy is provided by palmitic and stearic acids which are components of NEFA (Rukkwamsuk *et al*., 2000). However, a high concentration of NEFA in the blood results in high concentration of ketones in the plasma due to the inability of the liver to keep up with the oxidation of concentrated TAG in the blood stream. High concentrations of NEFA in the blood can also trigger the release of CCK that causes the cow to experience false satiety, resulting into reduced DMI and rumen digestion (Conrad *et al.,* 1964; Palmquist & Jenkins, 1980). Excessive NEFA are involuntarily stored in the liver and utilised in the β-oxidation pathway to form acetyl CoAs and NADH (Lean *et al*., 1992). During NEBAL, gluconeogenesis takes precedence over oxidation (Lean *et al*., 1992) which results in the accumulation of acetyl CoAs in the liver (Suriyasathaporn *et al*., 2000). Unfortunately, removal of acetyl CoAs is through conversion to ketone bodies or BHBA (Roche *et al*. 2009) which results in the development of fatty liver (Grummer, 1993; Van den Top *et al*., 1996).

Elevated NEFA is heavily associated with poor health and reproduction (Rukkwamsuk *et al*., 2000; Robinson *et al*., 2002; Leroy *et al*., 2005; Dillon *et al*. 2006; Roche *et al*., 2009; Rukkwamsuk *et al.,* 2010). In these studies, Vanholder et al. (2005) observed that increased concentrations of NEFA in the serum can cause cell apoptosis resulting in depressed granulosa cell proliferation and steroidogenesis. Supplementation of dairy cows with cis-*9, trans-11* and *trans-10, cis-12* CLA during early lactation can decrease the concentration of plasma NEFA (Odens *et al*., 2007). However, information regarding the mechanism by which dietary supplements rich in PUFA affect blood NEFA concentration is still scanty. Some proposed theories postulate that dietary fat supplement favours lower blood NEFA concentration by providing extra energy postpartum (Mattos *et al.,* 2000). Other research

findings indicate that feeding dairy cows with fat supplements could promote increased insulin and IGF-1 production (Blum *et al*., 1985; Baumgard *et al*., 2000; Lucy & Crooker, 2001). However, studies investigating the response of plasma insulin to fat supplementation are inconsistent. For instance, some studies reported decreased plasma concentrations of insulin, while others reported steady insulin increases postpartum in cows fed six different diets containing fats (Lucy *et al*., 1991). Addition of insulin to follicles and granulosa cells *in vitro* is known to increase cell proliferation and production of P4 (Lucy *et al*., 1993; Spicer *et al.,* 1993), hence, a nutritional strategy is required for the improvement of fertility parameters at the cost of greater milk production in dairy animals.

Milk Composition and Production Responses to Fat Supplementation

Bobe et al. (2009) fed mid-lactating dairy cows with tallow at 4.2% of dry matter (Mensink *et al*., 2003) and found that both milk fat and protein contents were increased. Chilliard et al. (2001) obtained similar results after they fed cows with ruminally inert tallow at 1.48 kg/cow/d. Chichlowski et al. (2005) fed ground canola seed to lactating Holstein cows and reported no change in milk yield, somatic cell count (SCC) and lactose percentage. However protein and fat percentages were lowered. Feeding multiparous Holstein-Friesians with encapsulated fat containing 40.8% flaxseed oil (E-FLAX) had no effect on their milk production, but encapsulated fat containing 40.8% sunflower oil (E-SUN) decreased milk production (Zachut *et al*., 2010). In the same study, milk fat percentage was significantly higher in the E-FLAX group than in the E-SUN group, although fat yield remained the same across treatment groups. The group of cows receiving E-FLAX produced less protein and had a lower protein percentage in their milk compared to the E-SUN and the control group. The E-FLAX and control groups produced milk with a higher lactose percentage but lower lactose yield than the E-SUN group. In another study by Caroprese et al. (2010), feeding whole flaxseed (FS) at 2.2 kg/cow/d and microencapsulated fish oil at 200 g/cow/d (FO) to Italian

Friesian cows did not affect milk yield, although higher numerical milk yield was obtained from the FS cows. In the same study, fat yield and fat percentage were significantly higher in the group receiving FS. However, no observable differences were seen between lactose, protein and SCC compositions across the different treatment groups. This result was also supported by the study of He and Armentano (2011), who found no change in milk protein yield of multiparous Holstein cows supplemented with palm oil, corn oil, linseed oil, high oleic and linoleic safflower oil. However, the overall milk protein concentration was significantly lower for the cows on linseed, palm oil, high oleic safflower and the control group. Corn oil and high linoleic safflower oil significantly lowered fat concentration and yield, while palm oil increased milk yield, fat yield and fat concentration. High linoleic safflower oil greatly decreased milk yield compared with linseed oil.

In a study using CLA, Hutchinson et al. (2012) reported significantly lower concentrations of milk fat and protein in both primiparous and multiparous lactating Holstein cows in Ireland, although milk yield increased significantly. In general, the differences in milk yield and milk composition between the different studies discussed above could be attributed to the different sources of lipid supplements and their fatty acid compositions. Observable differences in milk protein concentration have been reported by many authors to result from increasing or decreasing flow of rumen nitrogen to the duodenum, which upon absorption into the blood, increases plasma concentration of amino acids (Ikwuegbu & Sutton, 1982; Caroprese *et al*., 2010). An intravenous rumen infusion of sheep with linseed oil was reported to reduce protein digestibility but increased nitrogen flow to the duodenum (Ikwuegbu & Sutton, 1982).

Dairy cow supplementations with fat sources have been associated with milk fat depression (MFD; Caroprese *et al*., 2010). Plant oil that has been processed and containing *trans*-10, *cis*-12 CLA, has been shown to be a strong inhibitor of mammary milk fat synthesis (Baumgard *et al*., 2000; Kay *et al*., 2006; Shingfield & Griinari, 2007). Transcription factors responsible

for the up-regulation of lipogenic enzymes are regulated by steroyl response element binding protein 1 (SREBP-1; Harvatine & Bauman, 2006; Bauman *et al*., 2008). The expression of these genes was found to be down-regulated following supplementation with trans-10, cis-12 CLA (Baumgard et al., 2002).

The fatty acid composition of milk containing C18:0, C18:1, C18:2 and C18:3 were reported to increase when a diet containing these fatty acids was fed as a supplement to dairy cows (Petit *et al*., 2001; Chichlowski *et al*., 2005; Caroprese *et al*., 2010; Zachut *et al*., 2010). Hutchinson et al. (2012) found that supplementation of dairy cows with CLA significantly reduced the proportions of short and medium chain FA, although the proportions of C4:0, C14:0, C15:0 and C16:0 were not affected. Caroprese et al. (2010) found that cows receiving whole flaxseed had significantly lower proportions of C14:0, C15:0, C16:0 and C17:0 in their milk, while the proportions of C18:0, C18:1 and *cis*-9 MUFA were significantly higher. The decreased concentrations of short and medium chain FA and the increased concentrations of LC-PUFA in both milk and plasma would be beneficial to the reproductive success of cows and the carryover effect may be beneficial for humans through the consumption of bovine milk.

Fat Supplementation and Reproductive Function

In modern dairy farms, calving interval is used as a management tool to determine the reproductive success of dairy cows. A fertile cow is one which delivers a healthy calf on a yearly basis following successful conception and parturition (Ball & Peters, 2004). However, failure to successfully conceive may be due to cows not showing obvious signs of oestrus, no ovulation or delay in resumption of the ovarian cycle (Ranasinghe *et al*., 2011). Heifers are an important asset to the dairy farmer because they are the future replacement stock, and therefore are key determinants of the economic future of dairy farms. In order to identify the

success and failure of the reproductive performance of dairy cows, it is essential to understand the reproductive traits responsible for enhancing successful fertility and try to unravel the links with the fat component of a dairy cow's diet.

Oestrous cycle

The oestrus cycle occurs prior to ovulation and it is a rhythmic period in female bovines that is characterised by behavioural changes known as heat (Ball & Peters, 2004). Dairy cattle are polyestrus, meaning they can ovulate many times during the year (Bone, 1979). A typical female bovine oestrus cycle is characterised by an 18 to 24-day length period (Field & Taylor, 2008). The oestrus cycle has four discrete phases namely: proestrus, oestrus, metestrus and diestrus (Campbell *et al.,* 2003). Two organs namely the hypothalamus and anterior pituitary glands, which are located inside the cow's cranium, are essential for coordinating oestrous and other reproductive processes through the secretion of reproductive hormones (Ball & Peters, 2004). The chains of events of the oestrus cycle are controlled by reproductive hormones that are produced through the interaction of Graafian follicle, corpus lutea and hypothalamic-pituitary interrelationships (Ball & Peters, 2004). The availability and concentration of these hormones at the required site at the right moment are essential for the success of the reproductive performance of dairy cows over their lifetime (Field & Taylor, 2008). The oestrous cycle is initiated upon the release of gonadotropin releasing hormone (GnRH) from the hypothalamus (Bone, 1979; Campbell *et al.,* 2003). Gonadotropin releasing hormone stimulates the release of FSH and LH from the anterior pituitary gland (Ball & Peters, 2004). Gonadotropin releasing hormone and FSH enhance the recruitment of primary follicles that are stimulated by LH into antral, and then into mature follicles, which through the presence of FSH in the ovary can be ovulated (Webb *et al*., 2002). The presence of the dominant follicle is marked by the release of inhibin which causes the regression of the

remaining antral follicles (Webb *et al*., 2004). Inhibin also acts on the anterior pituitary gland to deploy a negative feedback mechanism that reduces the release of FSH (Parkinson, 2003).

Ovulation

Oestrogen (E2) production by the granulosa cells occurs pre-ovulation and is controlled by the action of insulin (Parkinson, 2003). The presence of E2 pre-ovulation is necessary to prepare the reproductive system for fertilisation by increasing the LH concentration (Ball & Peters, 2004). Inflammatory reaction stimulated by LH surge, makes thinner and ruptures the follicle wall to release the mature dominant follicle through the ovulatory process. LH surge is also responsible for the formation and initiation of the function and production of P4 of the corpus luteum (Juengel & Niswender, 1998). The ovum released during ovulation is dispensed into the oviduct and pushed downward through cilial movement of the oviduct (Ball & Peters, 2004).

Conception

The presence of P4 during the luteal phase inhibits the release of GnRH and LH through the negative feedback mechanism that it exerts on the hypothalamus and anterior pituitary gland (Field & Taylor, 2008). Progesterone (P4) is also responsible for the preparation of the uterus for conception (Webb *et al*., 1992; Senger, 1997). However, lack of fertilisation of released ova causes the corpus luteum to release a second hormone known as oxytocin (Wathes *et al.*, 1982). Oxytocin stimulates the release of $PGF_{2\alpha}$ from the endometrium (Field & Taylor, 2008) which causes the regression of the corpus luteum through luteolysis (Campbell *et al.*, 2003). If fertilisation occurs, the level of P4 remains high preventing $PGF_{2\alpha}$ production and supports pregnancy (Ball & Peters, 2004). The production of bovine interferone-tau (bINF-τ) increases in a pregnant cow and is used by the embryo to signal to the cow that conception has occurred (Campbell *et al.*, 2003).

Embryonic development

The syngamy of the male and female pronuclei within the cytoplasm of oocyte results in zygote formation (Sreenan *et al.,* 2001). Following fusion, continuous mitotic division of the zygote occurs and this creates a mass of unspecialised cells known as cell mass. Further division of the zygote results in a morula, which is a tight ball of cells formed between days 5-6 after fertilisation (Bone, 1979; Sreenan *et al.,* 2001). On approximately day six, the blastocyst is formed. The blastocyst consists of the trophoblast and inner cell mass. The latter is known to form the embryo while the former supplies the nutrients required by the cell mass (Senger, 1997; Ball & Peters, 2004). The beginning of the embryo phase is marked by the development of three germ layers (ectoderm, mesoderm and endoderm) on day 14 (Field & Taylor, 2008). The nervous system, hair, skin and hooves all arise from the ectoderm. The mesoderm is responsible for the formation of the heart, muscle and bones, while the endoderm ensures that the lining of the digestive tract and the respiratory tubes occurs (Field & Taylor, 2008). Maternal recognition of pregnancy is possible at day 16 when the embryo has satisfactorily developed (Ball & Peters, 2004). The embryo at this stage can rely on its own supply of nutrients from fluids within the uterine wall. However, in the long run, the embryonic disc develops into a transparent membrane that attaches onto the endometrium to allow uninterrupted flow of nutrients from the mother to the foetus and vice versa (Bone, 1979). However, it should be noted that the reproductive processes discussed above are for a typical normal dairy cow. Conversely, there are many fertility issues that are currently faced by the modern dairy cow.

Factors Affecting Reproductive Performance

The genetic progress in increasing milk production by dairy cows has led to a gradual but progressive decline in reproductive performance in diverse dairy production systems around the world. Selection for high milk yield followed by inadequate nutritional management and

large herd sizes have contributed tremendously to reduced fertility in the dairy industry for the past 20 years (Butler, 2003). Reproductive problems in the dairy industry arise as a result of insufficient nutrition, deleterious inherited genes and infection from microbes (Ball & Peters, 2004). Most developmental reproductive problems are associated with inheriting hazardous genes from either the sire or the dam. It is also possible that Deoxyribonucleic acid (DNA) damages during fertilisation or foetal development could result into reproductive failure in the resulting progeny. Two of the most commonly inherited diseases associated with cows are; bovine leukocyte adhesion deficiency (BLAD; Gerardi, 1996) and segmental aplasia of the Müllerian ducts (White heifer disease; Ball & Peters, 2004). The latter disease occurs when the gene for white coat colour hinders the development of the Müllerian ducts. Müllerian ducts also known as paramesonephric ducts are found in the developing embryo and subsequently develop into the uterus, cervix, oviduct and parts of the vagina (Ball & Peters, 2004). When interference of the development of the Müllerian ducts occurs, it results in blockage of the reproductive tracts in the progeny (Ball & Peters, 2004). Heterozygous individuals carrying the genes for BLAD tend to be infertile (Jánosa *et al.,* 1999). Reproductive failures can also occur as a result of infection of the reproductive system by invading pathogens such as bacteria, virus, fungus and protozoa (Ball & Peters, 2004). A well-known example of an infection of the uterus is endometritis (Sheldon *et al*., 2004). Endometritis is an inflammation of the uterus membrane specifically caused by *Campylobacter fetus* or non-specifically by *Escherichia coli* or *Campylobacter pyogenes* (Sheldon *et al*., 2004). The condition arises usually following the processes of artificial insemination (AI), dystocia and/or retained placenta (Ball & Peters, 2004).

Inactive ovaries

Dairy cows can only reproduce if the primary follicles develop to mature follicles (dominant) and go through ovulation. This is possible if the ovary is active postpartum. The ovaries of

cows are usually latent just after parturition because nature requires that the newly born calf must first be weaned before a new conception can occur (Capuco *et al*., 2001; Capuco *et al*., 2003). This sexual quiescence period can vary according to lactation, nutrition, farm management, environment, season, stress, suckling behaviour of the new born calf, the period taken to re-establish new ovarian activities and luteal activities (Royal *et al*., 2002). The acyclicity of dairy cows following parturition can be divided into physiological and pathological periods depending on the length of the sexual quiescence (Ball & Peters, 2004). The physiological period of acyclicity is usually between 30-40 days (Holmes *et al*., 2002) while pathological acyclicity occurs at day 50 and over (Ball & Peters, 2004). Physiological acyclicity also occurs to enable uterine involution (Ball & Peters, 2004). Pathological acyclicity mainly occurs due to lack of ovulation, the occurrence of an ovarian cyst or the inability of a healthy corpus luteum to regress (Mwaanga & Janowski, 2000). In a pasture-based system, acyclicity is the main factor causing reproductive problems in dairy cows and is correctly termed prolonged postpartum anovulatory interval (PPAI). Studies have shown that inadequate nutrition and NEBAL are to blame for PPAI (Beam & Butler, 1998). Beam and Butler (1997) and Canfield and Butler (1990), reported a negative relationship between ovulation and NEBAL in cows, suggesting that to improve the ovulating status of dairy cows, energy-dense supplements are needed in the diets of lactating cows.

Embryonic losses

Embryonic death can be experienced as early as day 24 or 25 of pregnancy (Ball & Peters, 2004). Cows and heifers raised and managed in a pasture-based system were found to have embryonic losses of 7.2% and 6.1%, respectively (Silke *et al*., 2002). Causes of embryonic loss are multifaceted and caused by nutritional, genetic, health, metabolic, hormonal and physiological factors (Ball & Peters, 2004). To date, nutrition seems to be the most important factor affecting foetal survival and other reproductive parameters of high merit cows. The

negative or positive energy status of a dairy cow postpartum depends on its pre-calving body condition score, which relates back to the availability and quality of available nutrition prepartum (Stockdale, 2000). Postpartum negative energy status of the modern dairy cow has always been implicated as the major problem affecting reproductive performance (Butler, 2003; Chagas *et al*., 2007).

Inbreeding, lethal genes and abnormal chromosomes have been found to heighten the loss of embryos (VanRaden & Miller, 2006). The ability of the embryo to signal to the dam its presence in the womb would prevent its loss. However, an under developed embryo at around day 24 of pregnancy may not be able to produce bIFN-τ, a signalling chemical indicating its presence in the uterus (Garrett *et al*., 1988). The correct hormonal interaction, particularly between $PGF_{2\alpha}$ and P4, is essential in maintaining and carrying the foetus to term without loss. López-Gatius et al. (2004) found that the addition of P4 to high yielding cows at the early embryonic growth stage reduces pregnancy loss.

Omega-3 Long Chain Polyunsaturated and Dairy Fertility

Infertility is considered a major problem in the dairy industry due to increasing number of services per conception, poor expression of oestrous signs, twinning and double ovulation (Lopez *et al,*. 2005). Omega-3 polyunsaturated fatty acids in dietary supplements offered to dairy cows positively influence fertility traits (Staples *et al.,* 1998; Santos *et al*., 2008). However, most of the studies have utilised rumen protected CLA, particularly in America, Europe and Ireland (Hutchinson *et al*., 2012). It has been reported that improved dairy fertility resulting from fat supplementation stems from the significant effect of specific FA and not from the provision of energy as previously thought (Mattos *et al.,* 2000). Most of the reproductive hormones are steroids and ω-3 LC-PUFA is responsible for the synthesis of steroid hormones. Several studies have investigated reproductive traits such as oocyte quality

and pre-ovulatory follicular growth in dairy cows using different sources of fat/oil containing ω-3 PUFA, but the results have been inconsistent and warrant further research. Robinson et al. (2002) observed an increase in medium sized follicle growth when experimental cows were supplemented with C18:2ω-6 or C18:3ω-3. Ponter et al. (2006) utilised soybean (concentrated ω-6 PUFA) and flaxseed (concentrated ω-3 PUFA) and found that the number of small follicles were lower in cows fed flaxseed than soybeans.

Omega-3 polyunsaturated fatty acids effects on reproductive hormones

Progesterone, E2 and $PGF_{2\alpha}$ (Ball & Peters, 2004) are essential dairy reproductive hormones. Oestrogen is involved with the preparation of the reproductive tracts for ova fertilisation and the initiation of pulsative surge of LH (Ball & Peters, 2004). Progesterone is the most important fertility hormone responsible for carrying pregnancy to term (Ball & Peters, 2004; Piccinato *et al.*, 2010), whereas $PGF_{2\alpha}$ counteracts the functions of P4 on the corpus luteum after failed fertilisation (Funston, 2004). The proposed mechanisms by which ω-3 PUFA affect reproductive hormones rely on their ability to regulate the production of $PGF_{2\alpha}$, and increase the availability of ovarian cholesterol (the main precursor for steroid hormones; Grummer & Carroll 1991; Staples *et al.*, 1998; Williams & Stanko, 2000; Funston, 2004). Some studies have reported a negative correlation between the consumption of rich dietary sources of ω-3 PUFA and plasma cholesterol concentration, which could potentially lead to lowered concentrations of P4 and E2 *in vivo* (Robinson *et al*., 2002; Mattos *et al*., 2004; Gulliver *et al*., 2012;). Cows fed sources of ω-6 PUFA are known to produce more cholesterol. The cholesterol can be utilised in the presence of steroidogenic acute regulatory (StAR) gene to synthesise P4 (Wang *et al*., 2000; Wathes *et al.*, 2007; Piccinato *et al*., 2010). Robinson et al. (2002) found that cows subjected to increasing concentration of ω-3 PUFA in their mid-luteal stage had low P4. Hinckley et al. (1996) found that low P4 in luteal cells was associated with increasing ω-3 PUFA. Steroidogenesis of reproductive hormones by ω-3

PUFA is a known phenomenon; however, mechanisms through which ω-3 PUFA modulate the synthesis of steroid hormones to affect the function of the ovary and the corpus luteum are largely unknown and warrant further elucidation. Conflicting findings amongst researchers investigating the effects of fat supplementation on dairy reproduction arise from use of different fat sources, numbers of animals and timing of the fat supplementation.

Luteinising hormone and follicles development

The availability of LH is paramount in the later stages of development and maturation of ovarian follicles (Ball & Peters, 2004). The future reproductive success of a dairy cow can only be determined by the availability and quality of the follicles produced (Funston, 2004). The pulsative secretion of LH and the production of primary follicles require the availability of sufficient energy which can be provided by fat supplementation (Funston, 2004). A review by Schillo (1992) of the detrimental effects of under-nutrition on LH pulse frequency proposed a mechanism by which fat exerts its effect on LH secretion through the increased production of propionate. Propionate is a precursor of glucose which exerts its effect on the anterior pituitary gland to foster the release of LH (Staples *et al.*, 1998). However, the mechanism by which fat supplementation affects LH secretion is still poorly understood and warrants further investigation (Mattos *et al.*, 2000).

The size and number of pre-ovulatory follicles are essential in determining the overall size of the corpus luteum (Gulliver *et al*., 2012). A large corpus luteum is known to produce more P4 which increases the rate of conception (Funston, 2004). Ambrose et al. (2006), Mendoza et al. (2011) and Petit et al. (2002) showed that a wider diameter of the corpus luteum and ovulatory follicles was possible to attain through supplementation of dairy cows with ω-3 PUFA. However previous studies have been conflicting. For instance, Homa and Brown (1992) reported a reduced follicle size following the consumption of ω-6 PUFA by dairy

cows. On the other hand, Zachut et al. (2010) found that feeding multiparous Holstein-Friesian cows diets rich in ALA caused increased production of small sized ovarian follicles, but diets rich in LA assisted in producing larger follicles. Other studies have reported the effect of ω-6 PUFA on follicle numbers, diameter and corpus luteum volume in dairy cows; however, there is a dearth of information on the effects of ω-3 PUFA on these parameters (Burke *et al*., 1996; Bilby *et al*., 2006).

Oocyte development

Follicular fluids comprising high concentrations of ω-3 PUFA have been shown by many authors to be essential for oocyte maturation (Fouladi-Nashta *et al*., 2009a; Zeron *et al.*, 2002). Fouladi-Nashta et al. (2009a) and Zeron et al. (2002) found that the addition of ω-3 PUFA from soybean, linseed and fish oil in cow diets improved oocyte maturation. However, high concentration of ω-6 PUFA in follicular fluids hinders mitosis, thereby reducing the maturation of oocytes (Marei *et al.*, 2010). A second experiment by Fouladi-Nashta et al. (2009b) did not find a significant impact of ω-3 and ω-6 PUFA on oocyte development. Assessment of the effect of ω-3 PUFA on oocyte growth, development and maturation is challenging because of lack of effective equipment that can maintain the integrity of oocytes throughout the experiments. Therefore, inconsistencies and or lack of adequate information on the effect of ω-3 PUFA on oocyte development are major challenges and more studies are needed.

Oestrous and ovulation

Burke et al. (1996) intravenously infused six mature Hampshire ewes with olive and soybean oil and found that ewes receiving olive oil had a shorter time to oestrus compared to the soybean group. The reason was because olive oil contains high proportions of monounsaturated fatty acids (ω-9) in comparison to soybean (Gulliver *et al*., 2012). The

interactions between ω-3 and ω-6 PUFA in the ovary have a huge impact on oestrus cycle and ovulation (Gulliver *et al*., 2012). The availability of ω-3 PUFA assists in the production of P4 which is responsible for corpus luteum formation and maintenance; while concentration of ω-6 PUFA in the follicular fluids enable the synthesis of $PGF_{2\alpha}$ hormone which initiates the luteolytic process that causes corpus luteum regression (Abayasekara & Wathes, 1999; Mattos *et al.,* 2000; Malau-Aduli *et al*., 2004b). Supplementation of dairy cows with oils provides energy that is essential for stimulating ovulatory processes (Gulliver *et al*., 2012). However, the effect of specific fatty acids on ovulation is not clear.

Embryo survival

A viable embryo implanted in the uterus of the cow should be able to send a signal to its dam to stop luteolysis (Spencer & Bazer, 2004). Maternal recognition of pregnancy is achieved when the embryo releases a chemical compound bIFN-τ that is recognised by the dam (Ball & Peters, 2004). Increasing the concentration of bIFN-τ in the cow's plasma prevents the expression of an oxytocin receptor which induces the release of $PGF_{2\alpha}$ hormone (Wathes & Lamming, 1995). Luteolytic processes occur with high concentrations of $PGF_{2\alpha}$ hormone in the endometrium and this affects the survival of the embryo (Thatcher *et al*., 1984). Omega-3 polyunsaturated fatty acids are known to inhibit the production of $PGF_{2\alpha}$ hormones and support the production of P4 hormone essential for the survival of the embryo (Inskeep, 2004; Childs *et al*., 2008). Results from Petit and Twagiramungu (2006) suggest that ω-3 PUFA formed from ALA may be responsible for embryo survival, although the result was not significant ($p=0.07$). Many *in vitro* studies have been conducted to establish the impact of ω-3 PUFA on embryo survival using bovine endometrial cells (BEND), however *in vivo* studies are needed to verify findings from the *in vitro* studies.

Fat Supplementation and Gene Expression

Located within animal cells is an organelle called peroxisome (Bone, 1979). This organelle is mainly found in the liver where it is responsible for fatty acid metabolism, and is activated by peroxisome proliferator (Kliewer *et al*., 2000). The presence of LC-PUFA *in vivo* corroborates the expression of genes responsible for fat metabolism (Sampath & Ntambi, 2004). Previously it was believed that production of eicosanoids and fluctuations of the cell membrane concentration of phospholipids were the main reason behind regulation of gene expression by fat (Jump *et al*., 2005). However, recently peroxisome proliferator-activated receptor (PPARs) was identified as the nuclear receptor that is activated by PUFA and that affects fat metabolism (MacLaren *et al*., 2006). Peroxisome proliferator-activated receptor have three isoforms; PPARα, PPARδ and PPARγ. A reproductive study by Froment et al. (2006) established that PPARs resides in reproductive tissues, and so this could be essential for regulating the effects of unsaturated fatty acid on dairy reproductive tissues. An *in vivo* study on beef heifers found that PPARδ and PPARα were expressed in the endometrium when cows were supplemented with ω-3 PUFA; this was confirmed by an *in vitro* study using BEND (MacLaren *et al*., 2006; Coyne *et al*., 2008). Expression of PPARα and PPARδ was confirmed in theca and stroma cells, while the PPARγ isoform was found in granulosa cells (Rees *et al.*, 2008).

Recently, lipids supplementation was reported to potentially play a crucial role in regulating the expression of genes essential for production and reproduction performances (Jeckel *et al*., 2014; Öner *et al*., 2014; Qi *et al*., 2014; Vahmani *et al.*, 2014). The expression profiles of reproductive and productive genes reported to be mostly affected by the dietary fat are Arylalkylamine N-acetyltransferase (AANAT; Perez *et al.*, 2010), B-cell translocation gene 2 (BTG2; Jeckel *et al*. 2014) and milk fatty acid synthase gene (FASN; Hussein *et al*., 2013). Arylalkylamine N-acetyltransferase (AANAT) is an essential gene for melatonin biosynthesis

(Jeckel *et al*., 2014). Melatonin is directly associated with optimal functioning of the ovary, where it regulates the hypothalamic-pituitary-gonadal axis to initiate folliculogenesis and steroidogenesis in quails (Chowdhury *et al.*, 2010) and rats (Fiske *et al*., 1984). B-cell translocation gene 2 (BTG2) is an anti-proliferative gene that regulates cell cycle growth (Choi *et al*., 2013), demonstrating that the anti-proliferative characteristic of BTG2 gene could be crucial during ovulation in mammals (Park *et al*., 2013). However, most BTG2 research investigations have focussed on cancer studies. The FASN gene is known to play a central role in *de novo* biosynthesis of fat in the mammary gland of mammals (Roy *et al*., 2006).

Previous studies found that that supplementation of lipid to mice down-regulated the expression of lipogenic genes in liver and adipose tissue, (Jump, 2002; Wang & Jones, 2004). The relative mRNA abundance of lipoprotein lipase (LPL), fatty acid synthase (FASN), sterol regulatory element-binding transcription factor 1 (*SREBF1*), and thyroid hormone responsive spot 14 (THRSP) were repressed in dairy cows given *trans*-10, *cis* 12 CLA (Harvatine *et al*., 2009). The suppression of FASN, stearoyl-CoA desaturase 1 (*SCD1*), and fatty acid desaturase 2 (FADS2) were also reported in dairy goats consuming dietary fat (Toral *et al*., 2013). The results of the previous studies suggest that dietary lipid supplemented to dairy cattle causes depression in milk fat and protein concentrations by suppressing the expression of fat related genes, however, limited studies have attempted to see the effect of fat supplementation on fertility related genes. Therefore, studies designed to explore the influence of dietary fat supplementation on the relative mRNA abundance of genes responsible for modification of lactation and fertility traits in the blood/tissues of dairy cattle are required in different production systems to enable informed choices and tailored decisions when feeding lactating cows with specific dietary fat supplements.

Knowledge gaps and Research Objectives

- Progesterone and prostaglandin hormones have been identified as limiting factors in the reproduction and fertility successes of dairy cows. Conflicting reports on the effects of ω-3 and ω-6 PUFA from dietary oil/fat supplementation upon progesterone and prostaglandin abound in published literature but there is scanty information on the effect of canola oil containing ω-3 PUFA on progesterone and prostaglandins in pasture-based dairy systems.

- A negative correlation exists between NEFA and reproductive traits in most dairy herds. This relationship is exacerbated by NEBAL and inadequate nutrition. There are inconsistent reports on the effect of fat supplementation on NEFA, BHBA and ketone bodies.

- Many researchers have reported the effect of supplementing dairy cows with differing sources of fat/oil on milk fatty acid composition, but the effect of canola oil supplementation on milk fatty acid profile of dairy cows in a pasture-based system has not yet been fully explored.

- Little attention has been given to investigating the relationships between fat supplementation and the immune response of dairy herds in pasture-based systems. Filling in this significant knowledge gap will assist dairy farmers operating under pasture-based settings to improve their health management techniques to enhance efficient reproduction and optimal profitability in their dairy system.

- A wide body of evidence exists that shows the effect of different sources of oil/fat supplementation on milk yield, milk composition, BCS and live weight in lactating dairy cattle but published empirical evidence of the impact of canola oil on these lactation parameters is lacking for dairy cows in pasture-based systems.

Therefore, the research objectives needed to address the identified knowledge gaps are;

- To investigate the relationship between the supplementation of dairy cows with ω-3 PUFA-containing canola and the associated BCS and live weight profiles of cows in pasture-based dairy systems.
- To evaluate the influence of ω-3 PUFA derived from canola oil on reproductive hormones (progesterone, oestrogen, prostaglandin, insulin-like growth factor-1, luteinising and follicle stimulating hormones).
- To investigate the fatty acid profile of milk from dairy cows supplemented with canola oil.
- To examine the effect of canola oil supplementation on plasma metabolites.
- To investigate the influence of canola oil supplementation on milk composition.

Conclusion

Omega-3 long chain polyunsaturated fatty acids have significant effect on reproductive success and general wellbeing of dairy cows mainly through PGF2, P4, E2, LH and FSH. Measurable reproductive parameters such as oestrus cycle, ovulation, embryo survival, parturition and calving interval have been strongly linked with high concentrations of ω-3 in the blood. However, there is a lack of information on the effect of ω-3 on dairy cow reproduction and fertility traits in pasture-based systems. Filling this knowledge gap could have long term positive implications for pasture based dairy industries. This literature review has also shown that specific ω-3 PUFA have a direct impact on reproduction and fertility traits in dairy cows. Fat supplementation may also provide extra energy postpartum capable of influencing lactation traits. The reproductive success of dairy cows in pasture-based systems will require early resumption of oestrus cycle postpartum, proliferation and ovulation of healthy oocytes, establishment of a healthy embryo and maintenance of pregnancy to term. Adequate and appropriate nutrition is required to allow high merit cows to continuously sustain increased milk production and maintain acceptable yearly calving patterns. Specific fatty acids found in supplemented fat can assist in the hormonal regulation essential for optimal reproduction and fertility.

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Chapter 3 : Effect of dietary supplementation of pasture-based primiparous Holstein-Friesian cows with crude degummed canola oil on body condition score, liveweight, milk yield and composition

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Abstract

The main objective of this study was to investigate the effect of incremental levels of CDCO supplementation to pasture-dominant diets of grazing, primiparous, Holstein-Friesian cows on lactation performance, milk composition and liveweight traits. We tested the hypothesis that supplementing primiparous Holstein-Friesian cows with CDCO in a pasture-based dairy system will increase milk yield, fat and protein contents, but decrease cow BCS and liveweight. A random allocation of twenty primiparous Holstein-Friesian cows into four treatments was utilised in an eight-week feeding trial after two weeks of adjustment. The experimental treatments included a wheat-based pellet without CDCO (control), wheat-based pellet with CDCO added at 25 mL/kgDM basis (low), 35 mL/kgDM basis (medium) and 50 mL/kgDM basis (high). Treatment and week (duration) of supplementation were significant sources of variation influencing milk yield ($P< 0.01$), fat ($P< 0.05$) and protein ($P< 0.001$). Cows in the high treatment group had the greatest milk yield (168.7±3.5 kg/week) and lower fat (3.3±0.1%) and protein (3.0±0.09%) than cows in the control group (milk yield of 157.1±3.5 kg/week, 4.0±0.2% fat and 3.1±0.0% protein). With the exception of somatic cell count and yield, the week (duration) of supplementation significantly influenced all milk composition traits. It was concluded that supplementation of grazing dairy cows with CDCO had no negative impact on BCS and body weight gain. CDCO can be used to enhance milk yield, but at the expense of milk fat and protein.

Keywords: milk yield; fat; protein; body condition score; liveweight; crude degummed canola oil

Introduction

Pasture is the main feed source in South Eastern Australia where dairy farms are mostly concentrated (Dairy Australia, 2011). In seasons where rainfall is below average, barley and wheat supplements are partially used in pasture-based systems to increase the energy intake and milk production of lactating cows (Akbaridoust *et al*., 2014). On a typical pasture-based dairy farm, primiparous cows are the most energy-challenged animals because they are at the bottom of the social hierarchy (Moran & McLean, 2001).

In spite of previous studies in other parts of the world suggesting that dietary fat supplements can increase milk yield (Griinari & Bauman, 2006; Bernal-Santos *et al*., 2010), such supplements are generally not very popular within the Australian dairy system mainly because of the associated costs and depression of milk fat and protein content (Khorasani *et al*., 1991; Wu & Huber, 1994; He & Armentano, 2011). The specific mechanism by which supplementary fat affects lactation traits is still largely unknown (Griinari & Bauman, 2006). Therefore, further studies in different dairy production systems are required to enable informed choices and tailored decisions when feeding lactating cows with specific dietary fat supplements, hence the need for the current study in a typical Australian pasture-based production system.

The effects of canola oil supplementation on primiparous Holstein-Friesian cows in the published literature are inconsistent and limited, particularly in pasture-based dairy systems. Therefore, we hypothesised that supplementing primiparous Holstein-Friesian cows in a pasture-based dairy system with CDCO will increase milk yield, fat and protein contents, but decrease cow BCS and liveweight traits. The main objective of this study was to investigate the effect of the dietary inclusion of incremental levels of CDCO for eight weeks to pasture-

dominant diets of grazing, primiparous Holstein-Friesian cows on lactation performance, milk composition and liveweight traits.

Materials and Methods

All experimental procedures were in accordance with the University of Tasmania Animal Ethics Committee guidelines, the 1993 Tasmania Animal Welfare Act and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Site and climatic conditions

The experiment was carried out at the University of Tasmania's Dairy Research Centre, Tasmanian Institute of Agriculture (TIA) Elliot Dairy Research Farm in Somerset, North-Western Tasmania, Australia, from September to November 2012. Tasmania is Australia's smallest state with a land size of 68,000 square kilometres and located within the cool, temperate, climatic zone at latitude 42˚ South and longitude 42˚145 East. It is characterized by four distinct seasons; winter, autumn, spring and summer. The experiment was carried out in spring when the mean annual rainfall and humidity were 2500 mm and 60%, respectively.

Animals Treatment and Experimental Design

The condition and energy status of the experimental cows was visually assessed based on BCS on a scale of 1-8 (DPI, 2003; Stockdale 2001). Twenty primiparous, spring-calving, purebred, Holstein-Friesian cows (average liveweight of 400 ± 40 kg, BCS 4 ± 1 and 40 ± 8 days in milk (DIM), were randomly allocated into 1 of 4 treatments of wheat-based pellet without CDCO (control), wheat-based pellet with CDCO added at 25 mL/kgDM (low), 35 mL/kgDM (medium) and 50 mL/kgDM (high). For the supplementation trial, a complete randomise experimental design (CRD) was applied. The current level of CDCO was calculated based on 7% total fat recommended for grazing cows (Schroeder et al., 2004). All the experimental animals were kept as a single herd in fenced paddocks under the same

grazing management with access to 3000 kgDMha^{-1} of a mixture of ryegrass (*Lolium perenne*), cocksfoot (*Dactylis glomerata*) and white clover (*Trifolium repens)* pasture. Water was offered *ad libitum.* Each cow received 6 kg of the pelleted supplements daily for eight weeks, after two weeks of adjustment. Supplements were offered to cows in two splits during morning (3 kg) and evening (3 kg) milking sessions at 05:00 h and 15:00 h. There were no orts from any of the treatment groups.

Feed chemical composition and analysis

Dry matter (DM) contents of the basal and experimental diets were determined by drying samples to a constant temperature at 65°C in a fan forced oven, finely ground to pass through a 2 mm sieve using Laboratory Mill (Thomas Model 4 Wiley® Mill; Thomas Scientific), and further dried at 105^0C for 24 h. The DM was computed as the difference between the initial and final weights of samples. Ash content was determined by combusting samples in a furnace at 600°C for 8 h. Neutral (NDF) and acid (ADF) detergent fibre contents were measured using an Ankom fibre analyser (ANKOM220; ANKOM Technology, USA). Nitrogen was determined using a Thermo Finnigan EA 1112 Series Flash Elemental Analyser and the values multiplied by 6.25 to give the crude protein (CP) percentage. Ether extract (EE) was determined using an Ankom fat/oil extractor (ANKOMXT15; ANKOM Technology, USA). Metabolisable energy was calculated as per Van Es (1975). The chemical compositions of control, treatment, and basal feeds are presented in Table 3.1

Table 3.1 Chemical composition of the experimental, control and basal feeds

Chemical composition (%DM)	Feeds		
	Control (No canola oil)	Treatment (canola oil)	Basal diet (Pasture)
MC	9.1	8.2	5.5
DM	90.9	91.8	94.5
ADF	9.0	8.0	27.7
NDF	21.1	20.0	45.9
EE	2.1	6.2	3.0
Ash	8.9	9.7	9.3
NFC	59.0	52.8	23.9
OM	91.1	90.3	90.7
CP	10.4	12.7	21.0
ME (MJ/kg DM)	4.07	4.08	3.99

All feeds were analysed based on a dry weight basis; Moisture content (MC), Dry matter (DM), organic matter (OM), neutral detergent fibre (NDF), acid detergent fibre (ADF), non-fibrous carbohydrate (NFC), ether extract (EE), crude protein (CP) and Metabolisable energy (ME). Treatment = the pooled values of feed with added canola oil (25 ml/ kgDM, 35 ml/kgDM and 50 ml/kgDM). Control = feed without canola oil, Basal diet = mainly mixed ryegrass pasture.

Milk sampling and analysis

Weekly milk samples were bulked from daily consecutive milkings at 05:00 h and 15:00 h for 8 weeks (2,240 samples in total). Representative aliquots of fresh milk samples from each cow were collected using the Milking Point Controller (MPC 680) fitted to a De Laval herringbone milking machine into labelled plastic vials containing bronopol blue milk preservative and stored at -20°C until further analysis (Kroger, 1985). No experimental cow suffered mastitis before, during or after the feeding trial period.

Fat, protein, lactose, solids non-fat, and somatic cell count (SCC) analyses were carried out at TasHerd Pty Ltd Hadspen, Tasmania, the officially contracted herd recording and milk testing agency, using the Fourier Transformed Infrared (FT-IR) spectrometry technology (Bentley Fourier Transform Spectrometer; BFTS). The weekly milk yield from each cow was recorded using De Laval's Alpro Herd Management System software (Alpro Windows 7.00 version 7.00.00, 2011). Fat-corrected milk (FCM) was computed using the equation below:

3.5% FCM = kg milk (0.4 + 0.15 fat %; Gaines & Davidson, 1923; cited from: Beever & Doyle, 2007)

Liveweight

Weekly liveweight of the cows was automatically recorded as they passed through the De Laval auto drafter (De Laval Automatic Weigh System AWS100). These weights were used to calculate the specific growth rate (SGR) = $100*[(ln W_1)-(ln W_0)]*D-1$, where W_0 and W_1 represent initial and final weights, and D is the duration of the experiment in days (Amirkolaie *et al*., 2005). Subjective assessment of BCS on a scale of 1-8 was also recorded weekly by the same assessor (DPI, 2003).

Statistical analyses

Initially, summary statistics by level and week of CDCO supplementation were computed to give means, standard deviations, standard error, variance, minimum and maximum values that were scrutinised for any data entry errors. Linear, cubic and quadratic orthogonal contrasts were tested by regressing the dependent on explanatory variables using PROC REG (SAS, 2009), but found to be inconsequential. Therefore, repeated measures analysis of variance in PROC MIXED (SAS, 2009) was employed fitting the fixed effects and second-order interactions of treatment and week of lactation, while base line milk values and cows were fitted as covariate and random effects, respectively. Prior to that, 1st-order autoregressive covariance structure was utilised. 1st-order autoregressive covariance structure was utilised because it has homogeneous variances and correlations that decline exponentially with distance i.e. variability in measurement is constant regardless of when you measure it. The degrees of freedom utilised in testing for significance and mean separation were estimated by the Satterthwaite method (SAS, 2009). Significant differences and mean separations at the $P < 0.05$ threshold were carried out using Tukey's probability pairwise comparison tests (SAS, 2009) and presented as LSM ±SEM for each treatment group.

Results

It was evident that CDCO supplementation significantly influenced milk yield ($P<0.01$), protein ($P<0.001$), fat ($P<0.05$) and solids non-fat ($P<0.05$) percentages (Table 3.2). Cows in the high treatment group receiving 50 mL/kgDM of CDCO produced greater milk yield (168.7±3.4 vs 157.1±3.7 kg/wk), but lower fat percentage (3.3±0.1 vs 4.0±0.2%) than unsupplemented cows in the control group (0 mL/kgDM; Table 3.2). Protein percentage was significantly ($P<0.001$) lower in the medium (35 mL/kgDM) treatment group than in the control and low treatment groups. Both treatment and week (duration) of supplementation had significant impacts on liveweight traits (BCS and SGR). Week of supplementation was a significant factor influencing almost all the lactation traits apart from somatic cell count ($P>0.05$), milk yield ($P>0.05$) and protein percentage ($P> 0.05$). The interaction between treatment and week of supplementation produced no significant effects on lactation and liveweight traits with the exception of BCS (Table 3.2).

Table 3.2 Least square means and standard errors (LSM ± SEM) of lactation and liveweight traits.

	Treatment				P-values		
Traits	Control	Low	Medium	High	Treatment	Week	Treatment*Week
MY	157.1±3.7^{c}	151.0±3.5^{dc}	162.9±2.9^{b}	168.7±3.4^{a}	0.0042	0.1204	1.0000
Fat%	4.0±0.2^{a}	3.7±0.1^{abc}	3.4±0.1^{bc}	3.3±0.1^{c}	0.0118	0.0043	0.5379
FY	0.8±0.0	0.8±0.0	0.7±0.0	0.8±0.0	0.8324	0.0045	0.3414
Protein%	3.1±0.0^{a}	3.2±0.1^{a}	2.9±0.0^{b}	3.0±0.0^{b}	0.0002	0.0768	0.9587
PY	0.7±0.0	0.7±0.0	0.7±0.0	0.7±0.0	0.3836	0.0019	0.4620
Lactose%	5.2±0.1	5.1±0.1	4.9±0.0	4.9±0.1	0.4380	0.0001	0.9798
LY	1.1±0.0	1.1±0.0	1.1±0.0	1.2±0.0	0.3842	0.0019	0.8499
SNF%	9.3±0.2^{a}	9.2±0.1^{ab}	8.7±0.1^{bc}	8.9±0.1^{b}	0.0136	0.0001	0.9521
SNFY	2.0±0.1	2.0±0.0	2.0±0.0	2.2±0.0	0.3750	0.0006	0.8834
SCC	54.5±9.2	121.4±24.0	56.3±9.3	84.5±46.9	0.3012	0.4718	0.7667
FCM	9.7±0.5	9.3±0.4	9.5±0.4	10.2±0.4	0.3687	0.0050	0.1219
SGR	2.2±0.6	2.2±0.6	2.2±0.6	2.2±0.6	0.2264	0.0001	0.0797
BCS	4.2±0.0	4.1±0.0	4.0±0.0	4.1±0.0	0.7729	0.0001	0.0020

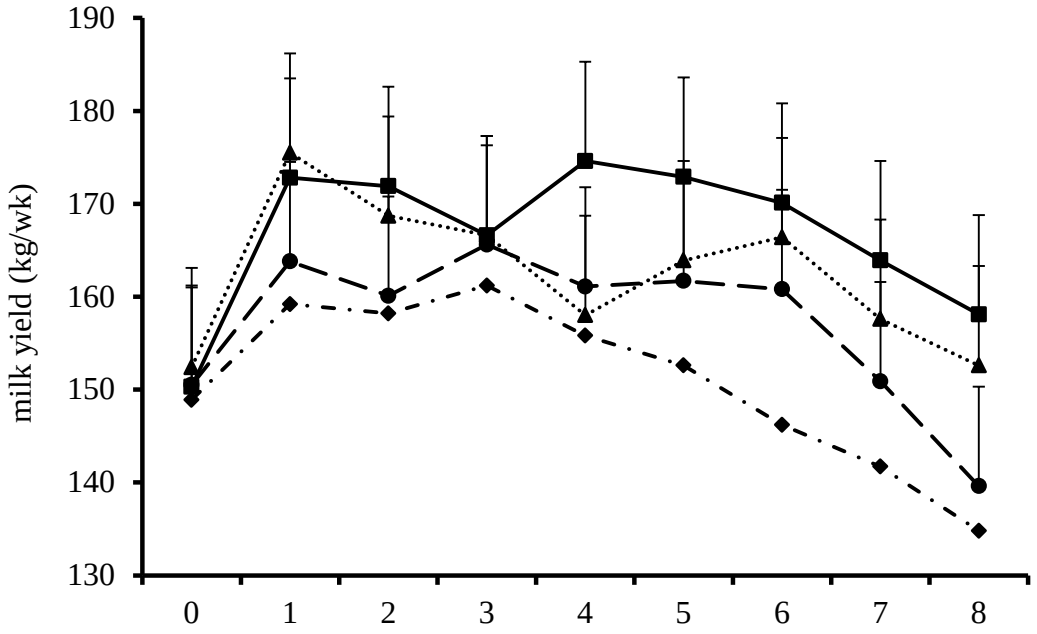
A mean bearing a different superscript (a, b, c) differs from the other means significantly (P<0.05). Milk yield (MY, kg/week), fat yield (FY, kg/d), protein yield (PY, kg/d), somatic cell count (SCC, x1000 cells/ml), lactose yield (LY, kg/d), solids non-fat yield (SNFY, kg/d), fat corrected milk (FCM, kg/d), specific growth rate (SGR, kg), BCS, Scale: 1-8).

Weekly trends for lactation and liveweight traits

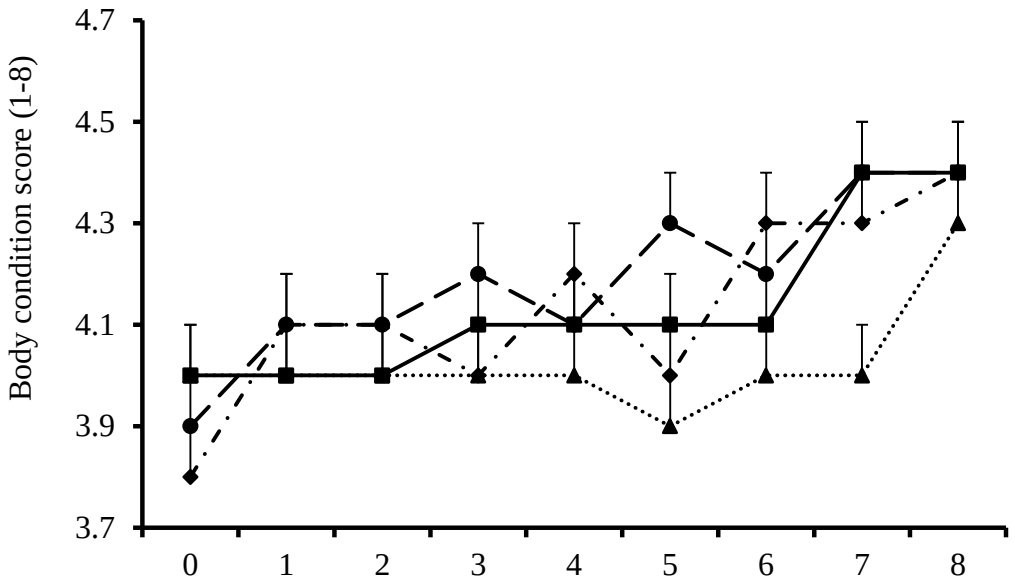
The peak milk yield for the medium and high occurred in week one and four, respectively, whereas, the control and the low group peaked in week three before tapering off in week seven and eight (Figure 3.1a). Body condition score for all the supplemented cows showed continuous increase throughout the duration of the feeding trial, although cows in the medium supplementation group had the least BCS (Figure 3.1b). The cows in the medium group had the highest fat percentage in week seven but petered off rapidly in week eight to below 3.5%. The control group yielded fat percentages greater than 4% in three occasions; in week two, five and seven, whereas the high group cows consistently produced low fat percentage

throughout the duration of the feeding trial (Figure 3.2a). Cows in the low treatment group had a peak fat yield in week five, but generally the fat yield between the groups were similar throughout the experimental period (Figure 3.2b). Cows in the low CDCO group consistently had the greatest protein percentage that rose from 3.1 % at the commencement of the feeding trial to 3.5 % in Week 8 (Figure 3.3a). However, the high treatment group yielded the highest (0.8 kg/d) protein in week five, with the low group producing the least amount of protein (0.5 kg/d). Throughout the trial period, the cows consuming 50 mL/kgDM generally produced more protein in milk than the rest of the groups (Figure 3.3b). The control group cows had a peak growth rate in week one and tapered off in week two. The high, medium and low group had peak growth rate in week one and decline to nadir in week four and six, respectively (Figure 3.1c).

(**a**)



(**b**)



(**C**)

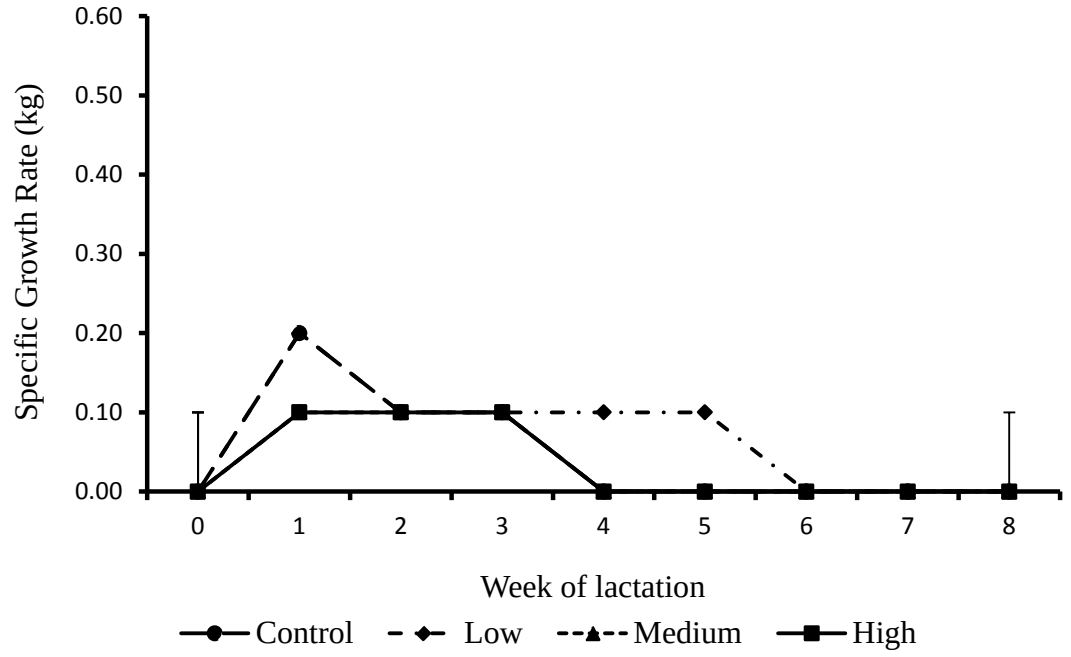
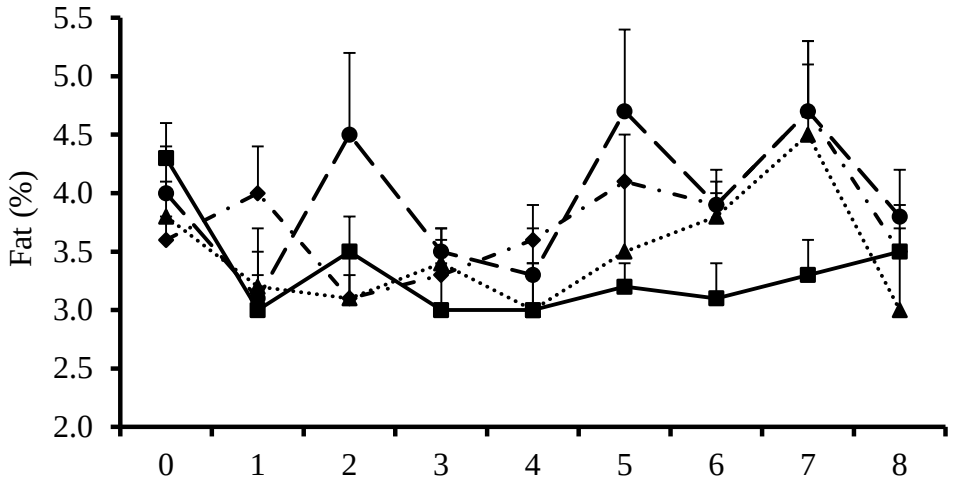


Figure 3.1 Weekly trends in milk yield (a), body condition score (b) and specific growth rate.

(**a**)



(**b**)

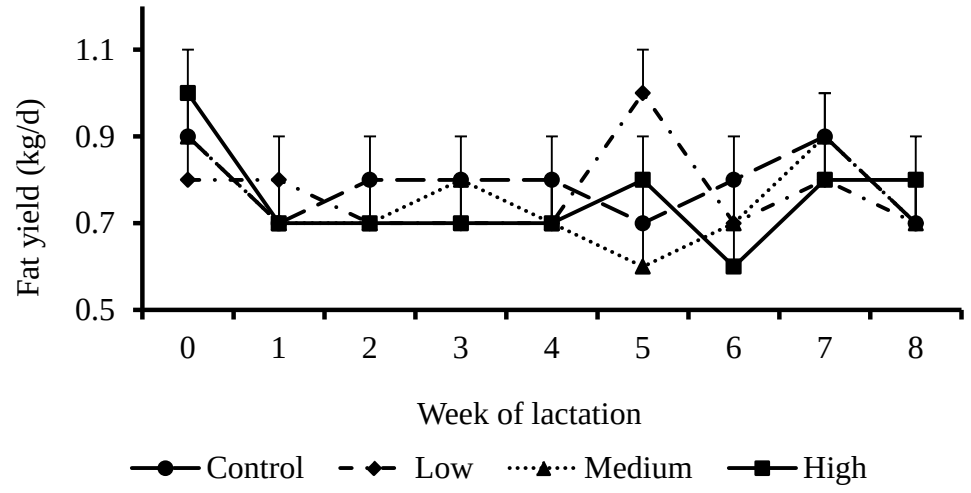
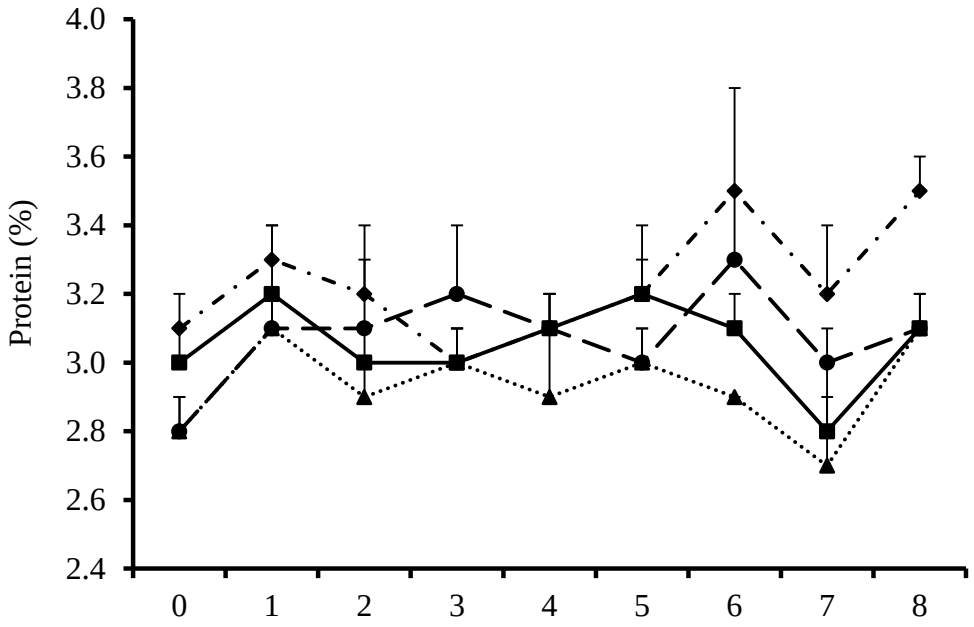


Figure 3.2 Weekly trends in milk fat percentage (a) and fat yield (b).

(**a**)



(**b**)

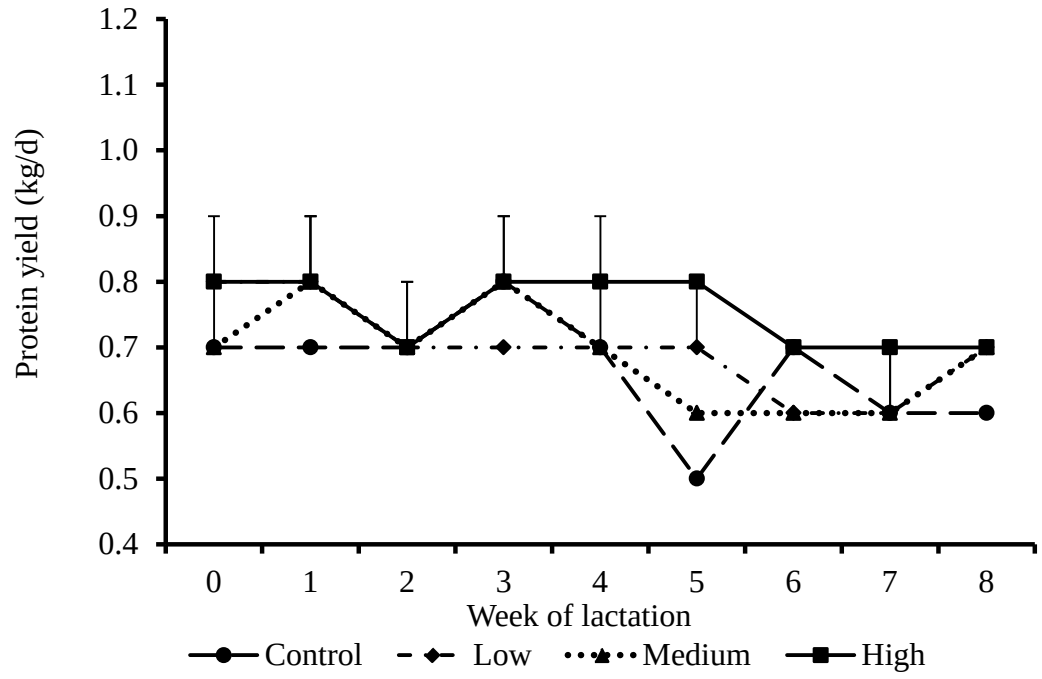


Figure 3.3 Weekly trends in milk protein percentage (a) and yield (b).

Discussion

Dietary supplementation of dairy cows with oils to increase milk production has been studied mostly in confined systems, where cows were fed total mixed rations, and conflicting findings were reported (Schroeder *et al*., 2004; Rabiee *et al*., 2012). Variation in results from a few pasture-based systems has also been observed (Kay *et al*., 2007; Hutchinson *et al*., 2011). The findings in this current study agree with previous authors who reported an increase in milk yield (Khorasani *et al*., 1991; Chilliard *et al*., 2001; Bobe *et al*., 2009; Hutchinson *et al.,* 2012), but contrasts with others who reported either a decrease or no effect on milk production (Bayourthe *et al*., 2000; Chichlowski *et al*., 2005; Bernal-Santos *et al*., 2010; Caroprese *et al*., 2010; He & Armentano, 2011) when fat sources rich in PUFA were included in the diet for cows. The increase in milk production could possibly be attributed to the greater energy density of the feed and better efficiency of energy utilisation (Jenkins & McGuire, 2006; Kay *et al*., 2007; Odens *et al*., 2007; He & Armentano, 2011). In addition, fat has the capacity to reduce heat and energy loss in urine, which potentially increases the efficiency of energy utilization and partitioning of absorbed nutrients for milk production and storage of excess energy in the adipose tissue of a lactating cow (Jenkins, 1993; Schroeder *et al*., 2004; Kay *et al*., 2007; Odens *et al*., 2007)

Our finding in this study is also in agreement with other reports that fat supplementation leads to a decrease in milk fat (Tackett *et al*., 1996; Bauman & Griinari, 2001, 2003; Peterson *et al*., 2003; Chichlowski *et al*., 2005; Griinari & Bauman, 2006; Hutchinson *et al*., 2012). Previous studies had linked differences in milk fat to protein ratio to genetic variation (Malau-Aduli & Anlade, 2002; Buttchereit *et al*., 2011; Buttchereit *et al*., 2012; Negussie *et al*., 2013). However, this needs further elucidation. Among other likely mechanisms involved in depressed milk fat and protein contents are the negative effects of fat supplementation on fibre digestion in the rumen leading to a reduction in the proportions of acetate to propionate

ratio, the main precursors of milk fat production (Schroeder *et al*., 2004). Secondly, fat percentage is influenced more by lipolytic processes that tend to change the fat to protein ratio in the milk depending on energy intake and rate of microbial protein synthesis (Negussie *et al*., 2013). Thirdly, a potent inhibitor of milk fat depression has been identified as *trans-10 cis-12 CLA*, which upon high concentration *in vivo* causes coordinated reduction of mRNA in the mammary gland responsible for activating primary enzymes for fat synthesis (Baumgard *et al*., 2000, 2002). Furthermore, milk fat yield is also largely dependent on, and intrinsically determined by milk yield because of the well-known negative correlation between milk fat content and milk yield (He & Armentano, 2011). Crude degummed canola oil has shown the potential to depress milk fat content, which at the same time, could be utilized effectively in postpartum grazing cows to improve cow body condition and energy status where dry matter intake is limited.

Milk fat and protein are the most economically important components of milk because of their contribution to total milk solids upon which dairy farmers are paid, but a negative relationship has been established between milk yield and fat (Chichlowski *et al*., 2005). Supplementation of cows with CDCO in this study marginally decreased milk protein concentration. Previous studies have reported decreased milk protein when fat was supplemented to dairy cows (Jahreis & Richter, 1994; Delbecchi *et al*., 2001; Larsen *et al*., 2012). The purported mechanism behind milk protein synthesis is associated with glucose deficit (Schroeder *et al*., 2004). Glucose provides the necessary energy rumen microbes need to drive the process of amino acid production necessary for milk protein synthesis (Wu & Huber, 1994). However, the physiological mechanism of how fat supplementation affects protein synthesis eludes us and warrants further investigation.

Crude degummed canola oil has shown the potential to depress milk fat content, which at the same time, could be utilized effectively in postpartum grazing cows to improve cow body

condition and energy status where dry matter intake is limited. Mammary gland synthesises milk fat using fatty acid from two sources; de novo and blood circulation. In this study, the observed milk fat depression is likely a result of CLA-rich dietary source reaching the mammary glands of dairy cows supplemented with CDCO. The depression in milk fat and increase in milk yield observed in the current study indicates that energy spared from milk fat synthesis is partitioned toward milk production rather than liveweight gains and reproductive performance.

Liveweight traits are regularly used in the dairy industry to estimate the energy status of dairy cows during lactation (Malau-Aduli & Abubakar, 1992; Roche *et al*., 2009; Stockdale, 2001). In the present study, the similar influence of treatment on liveweight traits suggests that supplementation of grazing dairy cows with CDCO had no negative impacts on BCS and body weight gain. Secondly, it also suggests the maintenance of a positive energy balance with limited depot fat remobilization from adipose tissues.

A good body condition score (4.5-5.4 on 8 point-scale) is critical for postpartum reproductive cyclicity. It has been reported that the most important management strategy to prevent postpartum anoestrous is to ensure that the average herd BCS is between 4.5 and 5.5 at calving (Kellaway & Harrington, 2004). In this study, the BCS of the experimental cows was within the required range; therefore, postpartum reproductive performance was not compromised. This is because the energy spared from reduced milk fat production was partitioned toward milk yield to avoid adipose tissue remobilisation. Over-conditioning of cows pre-calving (3-4 weeks prior) has negative impacts on reproductive performance (Van-den Top et al., 2005). Cows that are too fat at calving tend to mobilise adipose fat in large amounts, resulting in elevated NEFA in vivo (Van-den Top et al., 2005). Accumulation of NEFA in the blood and liver causes an increase in ketone bodies and fatty liver (Van-den Top et al., 2005). The effect of NEFA/ketone bodies on reproductive traits of dairy cows is well

established. Plasma NEFA has been associated with poor follicle and granulosa cell development (Beam & Butler, 1999). In the present study, similar influence of treatment on liveweight traits suggests that supplementation of grazing dairy cows with CDCO had no negative impacts on BCS and body weight gain. Secondly, it also suggests the maintenance of a positive energy balance with limited depot fat remobilization from adipose tissues.

Conclusion

This study showed that supplementing grazing primiparous Holstein-Friesian dairy cows with CDCO increased milk yield and depressed milk fat, without any negative impacts on BCS and liveweight gain. Depressed milk fat production could be useful in pasture-based systems where energy is limiting, to improve the energy status of cows for milk production. There is the need for further investigation into the underlying mechanisms with regard to circulating plasma metabolites and gene expression of supplemented cows to provide a better understanding of CDCO's role as a dietary fat supplement for lactating cows.

Acknowledgements

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Chapter 4 : Influence of supplementing pasture-based primiparous Holstein-Friesian dairy cows with crude degummed canola oil on milk fatty acid composition

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Abstract

The quest for alternative sources of healthy nutrients that facilitate the modification of milk without compromising drinking quality is a continuous research endeavour. The objective of the study was to quantify the milk fatty acid composition of pasture-based primiparous Holstein-Friesian dairy cows supplemented with CDCO with a view to improving the milk quality for beneficial health effects. This study tested the hypothesis that incremental supplementation of grazing primiparous Holstein-Friesian cows with CDCO will alter milk fatty acid composition towards increased total monounsaturates. Comparisons were made between unsupplemented grazing dairy cows and their peers on dietary supplements containing low (25 ml/KgDM), medium (35 ml/KgDM) or high levels (50 ml/KgDM) of CDCO in addition to *ad libitum* grazing access to pasture. There was no significant effect ($P>0.05$) of CDCO supplementation for eight weeks on the proportions of total polyunsaturated fatty acids (*t*PUFA), omega-3 (ω-3) and omega-6 (ω-6) fatty acids in milk. However, significant impacts of CDCO were observed on the proportions of 18:1ω9c, 18:1ω7t, total saturated (*t*SFA) and total monounsaturated (*t*MUFA) fatty acids ($P<0.005$), with a significant increase in the *t*MUFA/*t*SFA ratio in cows consuming CDCO. It was concluded that incremental levels of CDCO supplementation can modify the fatty acid composition of milk towards increased monounsaturates without any negative impact on grazing primiparous cows.

Keywords: monounsaturated fatty acids, polyunsaturated fatty acids, saturated fatty acids, omega-3, omega-6

Introduction

The demand for milk and other dairy products has slightly increased in Australia, with the consumption of drinking milk per capita rising from 104.4 litres in 2010/11 to 106.2 litres in 2011/12 (Dairy Australia, 2014). The primary focus of dairy farmers is to increase milk production with adequate fat and protein compositions because of the associated economic benefits of milk solids. In response to health concerns about coronary heart disease, obesity and arteriosclerosis, research interests in modifying milk fatty acid composition toward less saturated medium-chain (<C12) fatty acids and more LC-PUFA (>C18) are on the increase. The simplest way of altering milk fat composition is to supplement the diets of cows with unsaturated lipids (Glasser *et al.,* 2008; Hristov *et al*., 2011). Milk fat composition is changed more by the amount and composition of dietary fat than any other dietary component, and several studies (Palmquist *et al.,* 1993; Dewhurst *et al*., 2006; Chilliard *et al*., 2007) have been published on the response of milk fat composition to dietary lipid supplements in dairy cows. However, in Tasmania's pasture-based dairy production system, dietary supplementation of lactating cows with fat is not a common nutritional management practice, mainly because of its unknown impacts on milk fatty acid composition and other lactation traits. Previous fat studies in other dairy systems have reported the effects of fat supplements on milk fatty acid profiles (Chilliard *et al*., 2007; Glasser *et al.,* 2008; Hristov *et al*., 2011). Dietary fat supplementation of dairy cows in pasture-based production systems has been targeted toward enhancing the proportions of ω-3 and ω-6 PUFA at the expense of SFA to achieve desirable human health benefits (Simopoulos, 2008; Field *et al*., 2009; Shingfield *et al*., 2010). However, the beneficial health effect of fat supplements can be countered by the concurrent production of *trans*-MUFA known to be associated with cholesterol (Chardigny *et al*., 2008; Givens, 2010). Published studies in Australia investigating the impact of dietary fat

supplementation using CDCO on milk FA profiles of pasture-based primiparous cows are at best, scanty or non-existent, hence the need for this study to fill in the knowledge gap.

Canola oil products are readily available in Australia, and represent an excellent source of dietary fat, especially oleic acid (Khorasani *et al*., 1991; Ashes *et al*., 1992). However, extensive rumen biohydrogenation of canola can lead to the formation of *trans*-MUFA, an intermediate carbon-chain group of fatty acids that are undesirable for human consumption (Delbecchi *et al*., 2001). Therefore, information is required about the impact of supplementing lactating cows with CDCO on milk fatty acid composition. Furthermore, contrasting reports on the effect of canola supplementation on milk FA abound in the published literature, but there is a dearth of peered reviewed information on the use of CDCO as a supplement in pasture-based dairy production systems. However, studies conducted elsewhere using soybean and linseed oil reported an increase in the proportion of PUFA (C18:2 *cis*-9, 12 and C18:3 *cis*-9,12,15), whereas feeding cows with rapeseed oil decreased the proportion of MUFA (C18:1 *cis*-9) in milk fat (Jacobs *et al*., 2011).

Therefore in achieving this paper's objective, it was hypothesized that incremental supplementation of grazing primiparous Holstein-Friesian cows with CDCO will alter milk fatty acid composition towards increased total monounsaturates.

Materials and Methods

All experimental procedures were in accordance with the University of Tasmania (UTAS) Animal Ethics Committee guidelines, the 1993 Tasmania Animal Welfare Act and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Site and Climatic Conditions

The experiment was carried out in spring from September to November 2012 at the Dairy Research Centre of the Tasmanian Institute of Agriculture in Somerset, North-West of Tasmania, Australia, when the annual rainfall and humidity were approximately 2500mm and 60%, respectively. Tasmania is Australia's smallest state with a land size of 68,000 square kilometres and located within the cool, temperate, climatic zone at latitude 42° South and longitude 147° East characterized by four distinct seasons (winter, autumn, spring and summer).

Animals and Treatments

Body condition score of the cows was visually assessed on a scale of 1 to 8 (DPI, 2003; Stockdale, 2001). A total of 20 primiparous, spring-calving, and purebred Holstein-Friesian cows (average liveweight of 400 ± 40 kg, BCS4 ± 1 and 40 ± 8 DIM were randomly allocated into 1 of 4 treatments of CDCO (25 ml/kgDM, 35 ml/ kgDM and 50 ml/kgDM) and the control (no CDCO 0 ml/kgDM). For the supplementation trial, a complete randomise experimental design (CRD) was applied. All experimental cows (n=5 per treatment group) were placed under the same grazing management and rotated in electric-fenced paddocks. The control group of cows were offered wheat-based pellets with no CDCO and grazed on the same pastures comprising a mixture of ryegrass (*Lolium perenne*), cocksfoot (*Dactylis glomerata*), and white clover (*Trifolium repens)*. Water was offered *ad libitum.* The current level of CDCO was based on 7% total fat recommended for grazing cows (Schroeder *et al*., 2004). Supplements were offered to cows in two splits of 3 kg each during morning and

evening milking sessions at 05:00 h and 15:00 h, respectively, hence each cow received 6 kg of the pelleted supplement daily for eight weeks after two weeks of adjustment. There were no orts from any of the group of cows.

Feed Chemical Composition and Analysis

Dry matter (DM) content of the basal and experimental diets was determined by drying the samples to a constant temperature at 65°C in a fan forced oven, finely ground to pass through a 2 mm sieve using a Laboratory Mill (Thomas Model 4 Wiley® Mill; Thomas Scientific), and further dried at 105ºC for 24 h. The DM was computed as the difference between the initial and final weights of the samples. Ash content was determined by combusting the samples in a furnace at 600ºC for 8 hours. NDF and ADF fibre contents were measured using an Ankom fibre analyser ANKOM220; ANKOM Technology, USA. Total nitrogen was determined using a Thermo Finnigan EA 1112 Series Flash Elemental Analyser and the values multiplied by 6.25 to give the CP percentage. Ether extract was determined using an Ankom fat/oil extractor (ANKOMXT15; ANKOM Technology, USA). Metabolisable energy was calculated as per Van Es (1975). The chemical and fatty acid compositions of the treatment, control and basal feeds are presented in Tables 4.1 and 4.2.

Milk Sample Collection

Weekly milk samples were bulked from daily consecutive milkings at 05:00 h and 15:00 h for 8 weeks (2,240 samples in total). Representative aliquots of fresh milk samples from each cow were collected using the MPC 680 fitted to the De Laval herringbone milking machine into labelled plastic vials containing bronopol blue milk preservative and stored at -20ºC until further analysis (Kroger, 1985). No experimental cow suffered mastitis before, during or after the feeding trial period.

Table 4.1 Chemical composition of the experimental, control and basal feeds.

Chemical composition (%DM)	Feeds		
	Control (No canola oil)	Treatment (canola oil)	Basal diet (Pasture)
MC	9.1	8.2	5.5
DM	90.9	91.8	94.5
ADF	9.0	8.0	27.7
NDF	21.1	20.0	45.9
EE	2.1	6.2	3.0
Ash	8.9	9.7	9.3
NFC	59.0	52.8	23.9
OM	91.1	90.3	90.7
CP	10.4	12.7	21.0
ME (MJ/kg DM)	4.07	4.08	3.99

All feeds were analysed based on a dry weight basis; Moisture content (MC), Dry matter (DM), organic matter (OM), neutral detergent fibre (NDF), acid detergent fibre (ADF), non-fibrous carbohydrate (NFC), ether extract (EE), crude protein (CP) and Metabolisable energy (ME). Treatment = the pooled values of feed with added canola oil (25 ml/ kgDM, 35 ml/kgDM and 50 ml/kgDM). Control = feed without canola oil, Basal diet = mainly mixed ryegrass pasture.

Milk Fatty Acid Analysis

The milk samples were analysed using the gas-liquid chromatograph (GLC) method applied by the Commonwealth Scientific and Industrial Research Organization (CSIRO) Food Futures Flagship's Omega-3 Research Group, Marine and Atmospheric Research, Hobart, Tasmania, Australia, following direct methylation according to International Organization for Standardization (ISO) procedures. The procedure was as follows: Approximately 0.5 g of milk was freeze-dried and 0.05 mg of feed samples were weighed in duplicates into clean, 10ml screw-top methylation tubes and a freshly made solution of trans-esterification reaction mix (methanol:hydrochloric acid:chloroform (10:1:1 v/v/v, 3 ml) was added. Aliquots of milk were suspended in the transesterification solution and vortexed before trans-esterification at 80ºC for two hours. Each test tube was cooled for five minutes before 1ml of MilliQ water was added and the fatty acid methyl esters (FAME) were extracted using 3 ml x 2 ml of hexane:dichloromethane at a ratio of 4:1 v/v. Extracts from the methylation tubes were pipetted into vials, diluted with a known concentration of 19:0 FAME contained in

chloroform as the internal injection standard and were ready for gas chromatographic analysis. Chloroform was added to two vial tubes to form the blank controls for milk and feed samples. An Agilent Technologies 7890B GC equipped with a 15 m x 0.11 mm internal diameter cross-linked Equity-1 (0.1 um film thickness) fused-silica capillary column, a split/splitless injector, a 7683B series autosampler and flame ionization detector (Codabaccus *et al*., 2012) was used to analyse the FAME. Quantification of recorded peak areas was carried out using the software package Agilent Technologies Chemstation (Palo Alto, CA, USA). fatty acid methyl esters identity was confirmed by a GCQ (Thermoquest, USA) GC–mass spectrometer (GC–MS)', fitted with an on-column injector and an HP-5 cross-linked methyl silicone fused silica capillary column (50 m x 0.32 mm i.d.) of similar polarity to that described above. Quantification of recorded peak areas was carried out using the software package Millennium 32 v3.05.01 (Waters Corporation, USA). fatty acid methyl esters identity was confirmed by an MD 800 (Fissions, UK) or GCQ (Thermoquest, USA) GC–mass spectrometers (GC–MS) (Codabaccus *et al*., 2012). Quantified peaks were exported into an excel file, converted to total fatty acid percentages and subjected to statistical analysis.

Statistical Analysis

Initially, summary statistics by level and week of supplementation were computed to obtain means, standard deviations, standard error, minimum and maximum values which were closely scrutinised for any data entry errors. Subsequently, milk fatty acid composition was analysed by repeated measures analysis of variance (PROC MIXED) of SAS (2009) in which treatment, week of lactation and week of lactation by treatment interactions were fitted as fixed effects. Cow and baseline fatty acids values were fitted as random effects and covariate values, respectively. Prior to that, 1st-order autoregressive covariance structure was utilised. 1st-order autoregressive covariance structure was utilised because it has homogeneous variances and correlations that decline exponentially with distance i.e. variability in

measurement is constant regardless of when you measure it. Linear, quadratic and cubic contrasts were tested in regression analysis and found to have negligible impacts. Separation between means was conducted using Tukey's pairwise comparison and $P<0.05$ set as the threshold for significance.

Table 4.2 Fatty acid concentration as a percentage of total fatty acids of control, supplementary feeds and basal diets for lactating dairy cows.

Fatty acid (%)	Feed components		
	Control (No canola oil) %	Treatment (canola oil) %	Basal (Pasture) %
12:0	0.00	0.00	0.05
14:0	0.10	0.09	0.10
15:0	0.20	0.13	0.20
16:1	0.00	0.00	1.00
16:0	32.10	26.10	10.00
17:0	0.20	0.18	0.10
18:3ω 6	0.00	0.03	0.00
18:4ω 3	0.00	0.00	0.90
18:2ω 6 LA	17.70	6.86	9.10
18:3ω 3 ALA	1.60	0.48	64.30
18:1ω 9c	16.50	41.90	4.40
18:1ω 7t	0.20	0.10	0.20
18:0	3.80	3.83	2.20
18:2CLA	0.10	1.48	0.00
19:0	0.90	3.47	0.10
20:4ω 6 ARA	0.00	0.01	0.00
20:3ω 6	0.40	1.82	0.80
20:4ω 3 ETA	0.40	0.22	0.10
20:2ω 6	1.40	1.45	0.00
20:0	0.80	1.38	0.40
22:5ω 6	0.30	0.04	0.10
22:6ω 3 DHA	0.20	0.03	0.00
22:4ω 6	0.20	0.00	0.00
22:5ω 3 DPA	0.90	0.00	0.00
22:0	1.80	1.86	1.50
24:0	1.10	1.30	0.90
*t*SFA	41.20	38.64	16.45
*t*MUFA	23.30	48.74	8.00
*t*PUFA	35.00	12.62	75.40
ω-3 PUFA	14.90	0.93	65.40
ω-6 PUFA	20.10	10.24	10.10
ω-3 LC-PUFA	13.30	0.45	0.20
Other FA	11.80	0.20	0.10

∑*t*SFA is the sum of 12:0, 13:0, i14:0,14:0, i15:0, a15:0,15:0, i16:0, 16:0, i17:0, 17:0, i18:0, 18:0, 19:0, 20:0, 20:0, 22:0, 24:0; ∑*t*MUFA is the sum of 14:1ω -5c, 15:1ω-6c, 16:1ω -9c, 16:1ω -7c, 16:1ω -7t, 16:1ω -5c, 16:1,17:1ω -8+a17:0, 17:1ω-6c, 18:1ω -9c, 18:1ω -7c, 18:1ω -7t, 18:1ω -5c, 18:1a, 18:1b , 20:1ω -11c, 20:1ω -9c, 20:1ω -7c, 20:1ω -5c, 22:1ω -11c, 22:1ω -9c, 22:1ω -7c, 24:1ω -11c, 24:1ω -9c, 24:1ω -7c; ∑*t*PUFA is the sum of 18:3ω-6, 18:4ω-3, 18:2ω-6, 18:3ω-3, 18:2CLA, 20:4ω-6, 20:5ω-3, 20:3ω-6, 20:4ω-3, 20:2ω-6, 22:5ω-6, 22:6ω-3, 22:4ω-6, 22:5ω-3; ∑ω-3 LC-PUFA is the sum of 20:5ω-3, 20:4ω-3, 22:6ω-3, 22:5ω-3; ∑ω-3 PUFA is the sum of 18:4ω-3, 18:3ω-3, 20:4ω-3, 20:5ω-3, 22:6ω-3, 22:5ω-3; ∑ω-6 is the sum of 15:1ω-6, 17:1ω-6, 18:2ω-6, 18:3ω-6, 20:4ω-6, 20:3ω-6, 20:2ω-6, 22:5ω-6, 22:4ω-6. *t*SFA= total saturated fatty acids, *t*MUFA= total monounsaturated fatty acids, *t*PUFA= total polyunsaturated fatty acids, ω-3 FA= total omega-3 fatty acids, ω-6 FA=total omega-6 fatty acids, ω-3 LC-FA=total omega-3 long chain fatty acids, Other FA, Other FA= is the sum of unknown FA; Control= feed with no added canola oil; Treatment= feed with canola added; basal= mixed ryegrass pasture.

Results

Fatty acid Composition of Feedstuff

Table 4.2 shows that the CDCO supplement had higher proportions of 18:1ω9c, total monounsaturated fatty acids (*t*MUFA) compared to the control and basal diet. The control feed had greater proportions of 16:0, 18:2ω6 and *t*SFA compared to treatment and basal feed, whereas basal feed had higher proportion of 18:3ω3, *t*PUFA and ω-3 PUFA as compared to treatment and control feed. As expected, the CDCO supplement had higher proportions of 18:1ω9c, 18:2, 19:0, 20:3ω6, 20:0 and *t*MUFA, but less 18:2ω6, 18:3ω3 and *t*PUFA than both the control and pasture basal diets. The pasture basal diet also had the most ALA (18:3ω3) as expected.

Fatty Acid Composition of Milk

Canola oil supplementation level affected ($P<0.05$) some of the fatty acids (Table 4.3). Fatty acid profiles of the control and treatment groups were similar in some cases apart from: 14:0, 16:0, 18:0, 18:1ω9c, 18:1ω7t, 18:2CLAa, 18:2CLAb, 19:0, 20:3ω6, 20:2ω6, 20:0, 22:4ω6, 22:5ω3, 22:0, *t*SFA and *t*MUFA that differed between treatment groups. On the other hand, week of supplementation significantly affected: 18:1ω9c, 18:1ω7t, 18:2ω6, 18:ω3, *t*SFA and *t*PUFA (Table 4.4), whereas the interaction between treatment and week of supplementation yielded no effect on fatty acid profiles (Table 4.3 and 4.4).

Proportion of 18:1ω7t in Milk

Table 4.3 and 4.4 are results for the influence of treatment and week on fatty acid profiles, respectively. It was evident that level of CDCO supplementation was a significant source ($P<0.05$) of variation that influenced 18:1ω7t FA (Table 4.3). As the level of CDCO was increased, 18:1ω7t FA also increased in the milk. Cows in the high group produced the greatest 18:1ω7t percentage in comparison with the control group (7.5±0.5 vs 5.0±0.4), followed by the medium (6.0±0.3 vs 5.0±0.4) and low groups (5.7±0.5 vs 5.0±0.4),

respectively. Week also affected the concentration of 18:1ω7t FA in milk significantly (P<0.05), with highest (7.76±0.6) and lowest (4.39±0.4) levels yielded in week two and eight, respectively (Table 4.4). However, supplementation by week interaction had no significant effect (P>0.05: Table 4.3and 4.4).

Proportion of 18:1ω9c in Milk

Level (P<0.05) of supplementation of cows with CDCO significantly increased the concentration of 18:1ω9c in milk. The concentration of 18:1ω9c in both the high (19.6±0.6) and medium (19.6±0.6) treatment groups was similar, but higher (P<0.001) than the control (16.1±0.6) and low (16.4±0.5) groups (Table 4.3). Week of supplementation influenced the concentration of 18:1ω9c in milk significantly (P<0.05). Week zero (19.92±0.5) was highest followed by week eight (18.63±1.0) and lowest level was seen in week two (16.41±0.5: Table 4.4) However, the interaction between treatment and week of supplementation had no significant effect (P>0.05: Table 4.3and 4.4).

Proportions of tSFA and tMUFA in Milk

As the level of CDCO increased in the diet, the level of *t*SFA in the milk significantly decreased (P<0.05). The highest concentration of *t*SFA in milk was produced by the control group (64.94±1.2), followed by the low group (64.21±1.4: Table 4.3). The concentration of *t*MUFA was also significantly affected (P<0.05) by CDCO supplementation (Table 4.3). The high CDCO treatment group yielded the highest proportion of *t*MUFA (37.1±1.1vs30.2±1.0) compared to the other groups. Week had a significant (P<0.05) effect on *t*SFA concentration in milk (Table 4.4). However, week of supplementation and its interaction with treatment were not significant (P>0.05) sources of variation affecting the concentration of *t*MUFA (Table 4.4).

Proportion of tPUFA, ω-3 and ω-6 in Milk

Differences in CDCO content in the treatment groups had no significant effect on ω-3 and ω-6 FA. However, week of supplementation was a significant source of variation influencing *t*PUFA (Table 4.4).

Table 4.3 The mean fatty acid concentration (±SEM) (% total FA) of primiparous Holstein Friesian milk samples by level of supplementation with CDCO.

	Treatment group					P value	
Fatty acid (%)	Control	Low	Medium	High	RMSE	TRT	TRT*WK
14:0	14.36±0.3[b]	15.37±0.5[a]	13.62±0.5[c]	13.26±0.3[c]	1.94	***	NS
15:0	2.00±0.1	1.53±0.1	1.45±0.1	1.31±0.1	0.37	NS	NS
16:1	0.36±0.0	0.37±0.0	0.31±0.0	0.29±0.0	0.07	NS	NS
16:0	34.01±1.1[a]	31.97±1.7[a]	28.48±1.2[b]	27.49±0.9[b]	0.69	**	NS
17:0	0.69±0.0	0.57±0.0	0.59±0.0	0.56±0.0	0.10	NS	NS
18:3ω6	0.03±0.0	0.03±0.0	0.02±0.0	0.02±0.0	0.01	NS	NS
18:4ω3	0.03±0.0	0.03±0.0	0.05±0.0	0.06±0.0	0.04	NS	NS
18:2ω6	2.13±0.2	2.11±0.16	2.26±0.1	2.39±0.1	0.69	NS	NS
18:3ω3	0.83±0.1	0.85±0.1	0.95±0.1	0.97±0.1	0.53	NS	NS
18:1ω9c	16.09±0.6[b]	16.38±0.5[b]	19.64±0.6[a]	19.58±0.6[a]	2.84	***	NS
18:1ω7t	5.01±0.4[b]	5.69±0.5[b]	6.01±0.3[b]	7.49±0.5[a]	2.14	***	NS
18:0	7.16±0.5[b]	7.60±0.5[b]	9.28±0.5[a]	8.85±0.5[a]	2.09	***	NS
18:2CLAa	1.13±0.1	1.16±0.1	1.26±0.1	1.40±0.1	0.31	**	NS
18:2CLAb	0.25±0.0	0.24±0.0	0.26±0.0	0.29±0.0	0.08	*	NS
19:0	0.05±0.0	0.05±0.0	0.05±0.0	0.06±0.0	0.02	*	NS
20:5ω3	0.09±0.0	0.09±0.0	0.08±0.0	0.08±0.0	0.02	NS	NS
20:3ω6	0.08±0.0	0.08±0.0	0.07±0.0	0.07±0.0	0.02	*	NS
20:4ω3	0.04±0.0	0.04±0.0	0.04±0.0	0.04±0.0	0.02	NS	NS
20:2ω6	0.03±0.0	0.04±0.0	0.04±0.0	0.08±0.0	0.03	***	NS
20:0	0.09±0.0	0.10±0.0	0.11±0.0	0.13±0.0	0.04	***	NS
22:6ω3	0.01±0.0	0.01±0.0	0.01±0.0	0.00±0.0	0.01	NS	NS
22:4ω6	0.00±0.0	0.01±0.0	0.00±0.0	0.00±0.0	0.01	**	NS
22:5ω3	0.13±0.0	0.14±0.0	0.12±0.0	0.11±0.0	0.03	*	NS
22:0	0.05±0.0	0.05±0.0	0.06±0.0	0.05±0.0	0.01	*	NS
24:0	0.02±0.0	0.01±0.0	0.01±0.0	0.01±0.0	0.01	NS	NS
Σ*t*SFA	64.94±1.2[a]	64.21±1.4[a]	59.48±1.1[b]	57.33±1.1[b]	6.17	***	NS
Σ*t*MUFA	30.18±1.0[b]	30.87±1.1[b]	35.27±1.0[a]	37.08±1.1[a]	5.39	***	NS
Σ*t*PUFA	4.88±0.3	4.92±0.3	5.25±0.3	5.59±0.2	1.35	NS	NS
Σω-3 PUFA	1.12±0.1	1.15±0.1	1.24±0.1	1.26±0.1	0.55	NS	NS
Σω-6 PUFA	2.37±0.2	2.38±0.2	2.48±0.2	2.65±0.1	0.72	NS	NS
Σω-3LC-PUFA	0.27±0.0	0.27±0.0	0.25±0.0	0.23±0.0	0.06	NS	NS

Values with different superscript are significantly different; ∑*t*SFA is the sum of 12:0, 13:0, i14:0,14:0, i15:0, a15:0,15:0, i16:0, 16:0, i17:0, 17:0, i18:0, 18:0, 19:0, 20:0, 20:0, 22:0, 24:0; ∑*t*MUFA is the sum of 14:1w-5c, 15:1ω-6c, 16:1ω-9c, 16:1ω-7c, 16:1ω-7t, 16:1ω-5c, 16:1,17:1ω-8+a17:0, 17:1ω-6c, 18:1**ω**-9c, 18:1ω-7c, 18:1ω-7t, 18:1ω-5c, 18:1a, 18:1b , 20:1ω-11c, 20:1ω-9c, 20:1ω-7c, 20:1ω-5c, 22:1ω-11c, 22:1ω-9c, 22:1ω-7c, 24:1ω-11c, 24:1ω-9c, 24:1ω-7c; ∑*t*PUFA is the sum of 18:3ω-6, 18:4ω-3, 18:2ω-6, 18:3ω-3, 18:2CLAa, 18:2CLAb, 20:4ω-6, 20:5ω-3, 20:3ω-6, 20:4ω-3, 20:2ω-6, 22:5ω-6, 22:6ω-3, 22:4ω-6, 22:5ω-3; ∑ω-3 LC-PUFA is the sum of 20:5ω-3, 20:4ω-3, 22:6ω-3, 22:5ω-3; ∑ω-3 PUFA is the sum of 18:4ω-3, 18:3ω-3, 20:4ω-3, 20:5ω-3, 22:6ω-3, 22:5ω-3; ∑ω-6 is the sum of 15:1ω-6, 17:1ω-6, 18:2ω-6, 18:3ω-6, 20:4ω-6, 20:3ω-6, 20:2ω-6, 22:5ω-6, 22:4ω-6;*t*SFA= total saturated fatty acids; *t*MUFA= total monounsaturated fatty acids; *t*PUFA= total polyunsaturated fatty acids;ω-3 FA= total omega-3 fatty acids;ω-6 FA=total omega-6 fatty acids;ω-3 LC-FA=total omega-3 long chain fatty acids; NS = no significance; * = significant (P<0.05); ** = highly significant (P<0.01); *** = very highly significant (P<0.001);Treatment group= group of cows receiving canola oil; TRT=treatment feed, WK= Week, RMSE = root mean square error.

Table 4.4 The mean fatty acid concentration (±SEM) (% total FA) of primiparous Holstein Friesian milk samples by duration (week) of supplementation with CDCO.

	Week of supplementation							P values	
Fatty acid (%)	0	2	4	6	7	8	RMSE	WK	TRT*WK
14:0	12.45±0.3	14.19±0.3	14.03±0.4	15.67±0.6	13.77±0.5	14.80±0.6	1.94	NS	NS
15:0	1.22±0.0	1.85±0.1	1.76±0.1	1.60±0.1	1.53±0.1	1.49±0.1	0.37	NS	NS
16:1	0.25±0.0	0.35±0.0	0.36±0.0	0.33±0.0	0.35±0.0	0.35±0.0	0.07	NS	NS
16:0	26.88±0.8	28.72±1.0	29.54±1.4	31.76±2.3	32.23±1.4	33.79±2.2	0.69	NS	NS
17:0	0.60±0.0	0.64±0.0	0.62±0.0	0.58±0.0	0.59±0.0	0.58±0.0	0.10	NS	NS
18:3ω6	0.02±0.0	0.02±0.0	0.02±0.0	0.03±0.0	0.03±0.0	0.02±0.0	0.01	NS	NS
18:4ω3	0.02±0.0	0.05±0.0	0.03±0.0	0.03±0.0	0.05±0.0	0.05±0.0	0.04	NS	NS
18:2ω6	2.58±0.1^{a}	2.59±0.1^{a}	2.73±0.2^{a}	2.08±0.2^{b}	1.94±0.2^{b}	1.41±0.2^{c}	0.69	***	NS
18:3ω3	1.11±0.0^{a}	1.22±0.1^{a}	1.10±0.1^{a}	0.91±0.1^{ab}	0.74±0.1^{b}	0.32±0.1^{c}	0.53	***	NS
18:1ω9c	19.92±0.5^{a}	16.41±0.5^{bc}	17.35±0.7^{bc}	16.82±0.9^{bc}	18.41±0.8^{ab}	18.63±1.0^{ab}	2.84	**	NS
18:1ω7t	6.55±0.5^{ab}	7.76±0.6^{a}	6.70±0.6^{ab}	5.35±0.5^{bc}	5.55±0.5^{bc}	4.39±0.4^{c}	2.14	***	NS
18:0	11.96±0.4	7.79±0.3	6.86±0.4	7.50±0.7	7.59±0.6	7.63±0.6	2.09	NS	NS
18:2CLAa	1.07±0.0	1.25±0.1	1.37±0.1	1.32±0.1	1.36±0.1	1.06±0.1	0.31	NS	NS
18:2CLAb	0.24±0.0	0.28±0.0	0.27±0.0	0.21±0.0	0.25±0.0	0.31±0.0	0.08	NS	NS
19:0	0.06±0.0	0.05±0.0	0.05±0.0	0.05±0.0	0.04±0.0	0.05±0.0	0.02	NS	NS
20:5ω3	0.10±0.0	0.07±0.0	0.09±0.0	0.08±0.0	0.08±0.0	0.09±0.0	0.02	NS	NS
20:3ω6	0.08±0.0	0.07±0.0	0.08±0.0	0.07±0.0	0.08±0.0	0.08±0.0	0.02	NS	NS
20:4ω3	0.06±0.0	0.04±0.0	0.04±0.0	0.03±0.0	0.04±0.0	0.04±0.0	0.02	NS	NS
20:2ω6	0.06±0.0	0.05±0.0	0.06±0.0	0.03±0.0	0.04±0.0	0.04±0.0	0.03	NS	NS
20:0	0.14±0.0	0.10±0.0	0.10±0.0	0.10±0.0	0.10±0.0	0.11±0.0	0.04	NS	NS
22:6ω3	0.01±0.0	0.00±0.0	0.01±0.0	0.01±0.0	0.00±0.0	0.01±0.0	0.01	NS	NS
22:4ω6	0.00±0.0	0.00±0.0	0.00±0.0	0.01±0.0	0.01±0.0	0.01±0.0	0.01	NS	NS
22:5ω3	0.12±0.0	0.11±0.0	0.13±0.0	0.13±0.0	0.13±0.0	0.15±0.0	0.03	NS	NS
22:0	0.06±0.0	0.05±0.0	0.05±0.0	0.05±0.0	0.05±0.0	0.06±0.0	0.01	NS	NS
24:0	0.02±0.0	0.01±0.0	0.01±0.0	0.01±0.0	0.01±0.0	0.02±0.0	0.01	NS	NS
Σ*t*SFA	59.43±1.1^{a}	59.62±1.4^{a}	59.78±1.7^{a}	64.03±1.8^{a}	61.73±1.7^{a}	64.35±1.7^{a}	6.17	*	NS
Σ*t*MUFA	35.02±0.9	34.55±1.2	34.19±1.5	30.94±1.6	33.44±1.5	31.97±1.5	5.39	NS	NS
Σ*t*PUFA	5.55±0.2^{abc}	5.83±0.3^{ab}	6.03±0.4^{a}	5.03±0.3^{bc}	4.83±0.3^{c}	3.68±0.3^{d}	1.35	***	NS
Σω-3 PUFA	1.41±0.1	1.49±0.1	1.40±0.2	1.19±0.1	1.03±0.1	0.64±0.1	0.55	NS	NS
Σω-6 PUFA	2.83±0.1	2.82±0.2	2.99±0.2	2.31±0.2	2.19±0.2	1.67±0.2	0.72	NS	NS
Σω-3LC-PUFA	0.28±0.0	0.22±0.0	0.26±0.0	0.25±0.0	0.24±0.0	0.27±0.0	0.06	NS	NS

Values with different superscript are significantly different; ∑*t*SFA is the sum of 12:0, 13:0, i14:0,14:0, i15:0, a15:0,15:0, i16:0, 16:0, i17:0, 17:0, i18:0, 18:0, 19:0, 20:0, 20:0, 22:0, 24:0; ∑*t*MUFA is the sum of 14:1w-5c, 15:1ω-6c, 16:1ω-9c, 16:1ω-7c, 16:1ω-7t, 16:1ω-5c, 16:1,17:1ω-8+a17:0, 17:1ω-6c, 18:1**ω**-9c, 18:1ω-7c, 18:1ω-7t, 18:1ω-5c, 18:1a, 18:1b , 20:1ω-11c, 20:1ω-9c, 20:1ω-7c, 20:1ω-5c, 22:1ω-11c, 22:1ω-9c, 22:1ω-7c, 24:1ω-11c, 24:1ω-9c, 24:1ω-7c; ∑*t*PUFA is the sum of 18:3ω-6, 18:4ω-3, 18:2ω-6, 18:3ω-3, 18:2CLAa, 18:2CLAb, 20:4ω-6, 20:5ω-3, 20:3ω-6, 20:4ω-3, 20:2ω-6, 22:5ω-6, 22:6ω-3, 22:4ω-6, 22:5ω-3; ∑ω-3 LC-PUFA is the sum of 20:5ω-3, 20:4ω-3, 22:6ω-3, 22:5ω-3; ∑ω-3 PUFA is the sum of 18:4ω-3, 18:3ω-3, 20:4ω-3, 20:5ω-3, 22:6ω-3, 22:5ω-3; ∑ω-6 is the sum of 15:1ω-6, 17:1ω-6, 18:2ω-6, 18:3ω-6, 20:4ω-6, 20:3ω-6, 20:2ω-6, 22:5ω-6, 22:4ω-6;*t*SFA= total saturated fatty acids; *t*MUFA= total monounsaturated fatty acids; *t*PUFA= total polyunsaturated fatty acids;ω-3 FA= total omega-3 fatty acids;ω-6 FA=total omega-6 fatty acids;ω-3 LC-FA=total omega-3 long chain fatty acids; NS = no significance; * = significant (P<0.05); ** = highly significant (P<0.01); *** = very highly significant (P<0.001); week of supplementation= weeks when cows were fed with canola oil; TRT=treatment feed, WK= Week, RMSE = root mean square error.

Weekly Fatty Acid Composition Values

As the level of canola oil increased in the diet, weekly 18:1ω7t FA also increased (Figure 4.1). Cows in the high oil treatment group produced the greatest 18:1ω7t, rising from 6.7% to a peak of 9.8% in week two before tapering off to 5.0% at the end of the feeding trial in week eight. The other treatments were characterised by inconsistent rise and fall in values across week with the control and low treatment groups having the lowest values at week eight. Milk 18:1ω9c FA concentration increased in the milk of cows receiving medium and high levels of CDCO in the diet (Figure 4.2). Cows in the control and low treatment groups consistently had the least milk 18:1ω9c FA concentration trends throughout the trial period. Cows receiving the high and medium CDCO diets consistently produced milk with lower total *t*SFA percentage (Figure 4.3) compared to the control treatment, whereas the cows in the control and low CDCO treatment groups had the greatest weekly values of *t*SFA. Cows in the high CDCO treatment group had the greatest milk concentration of *t*MUFA from week two through eight (Figure 4.4). After the fourth week, the decline in the values of *t*MUFA of cows receiving control and low CDCO diet was so marked compared to the initial values at the start of the trial. Total polyunsaturated fatty acids (Figure 4.5), ω-6 (Figure 4.6) and ω-3 (Figure 4.7) values were consistently similar for all the treatment groups from weeks zero through eight.

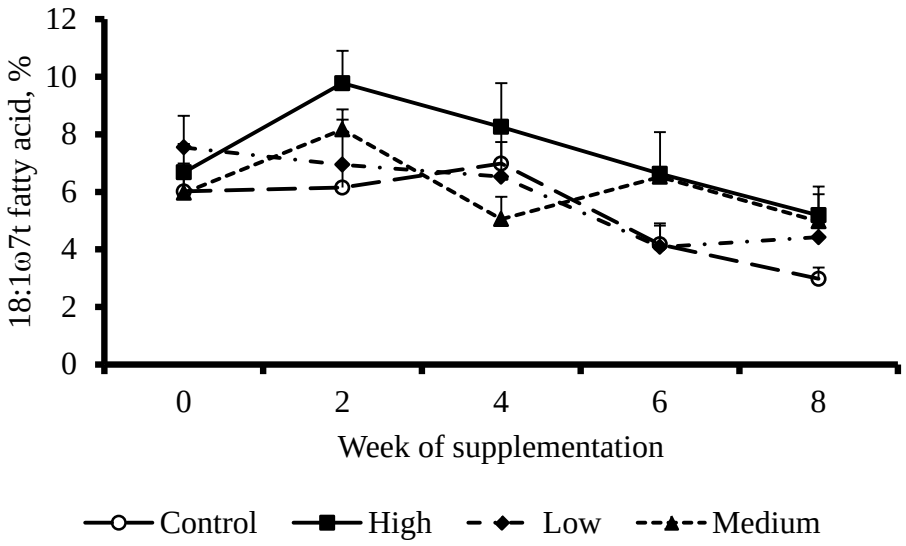

Figure 4.1 Weekly proportions of 18:1ω7t in milk of cows supplemented with varying levels of CDCO in the diet.

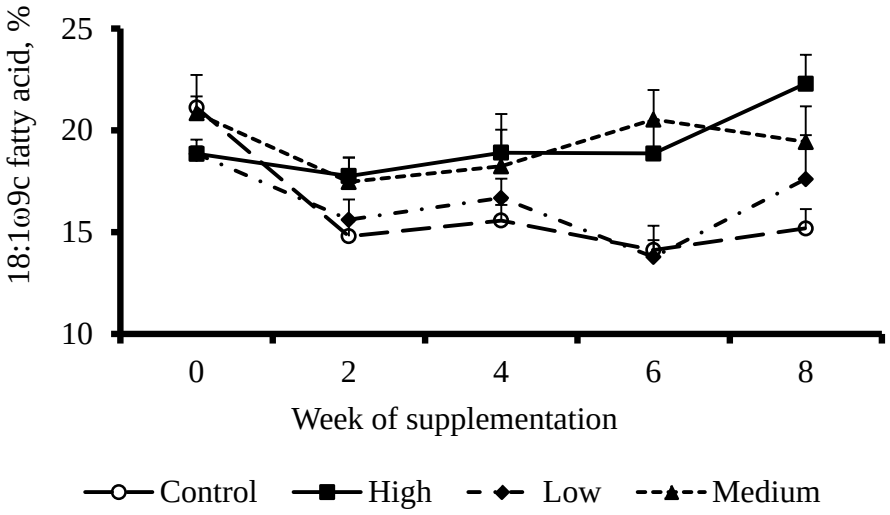

Figure 4.2 Weekly proportions of 18:1ω9c in milk of cows supplemented with varying levels of CDCO in the diet.

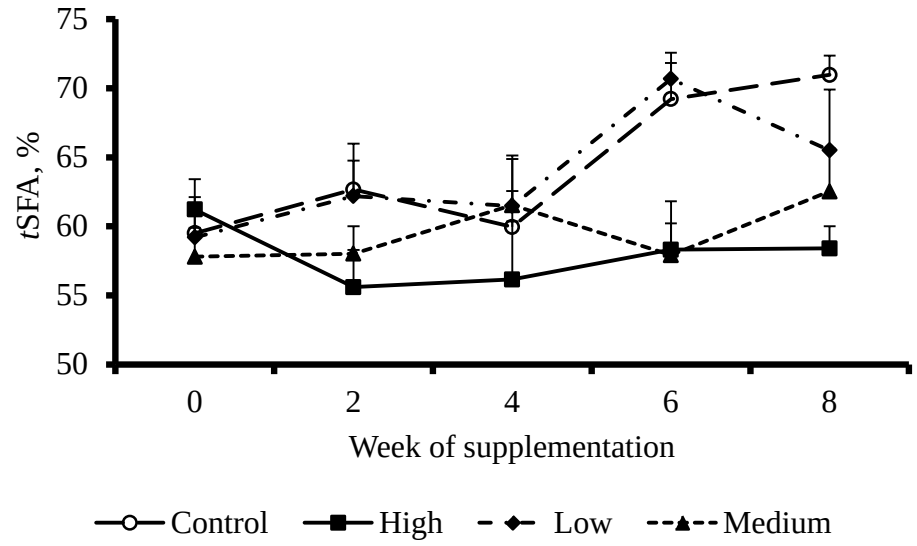

Figure 4.3 Weekly proportions of total saturated fatty acid (*t*SFA) in milk of cows supplemented with varying levels of CDCO in the diet.

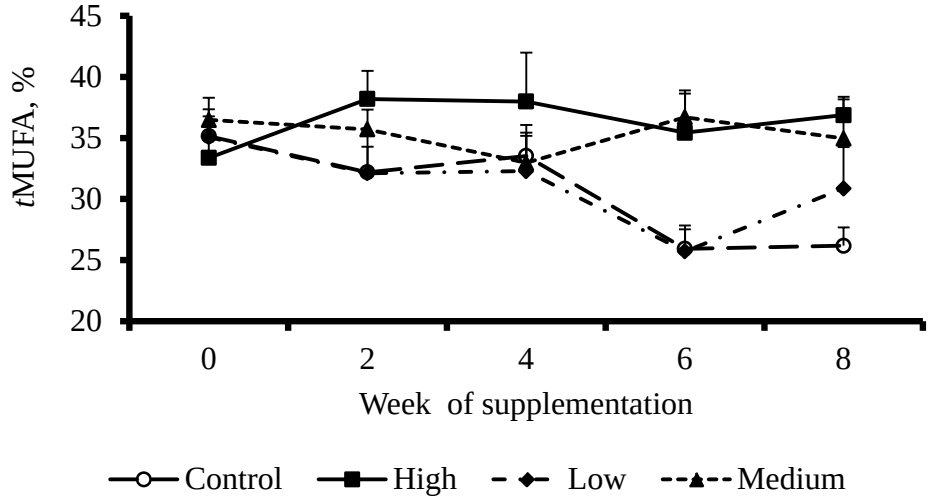

Figure 4.4 Weekly proportions of total monounsaturated fatty acid (*t*MUFA) in milk of cows supplemented with varying levels of CDCO in the diet.

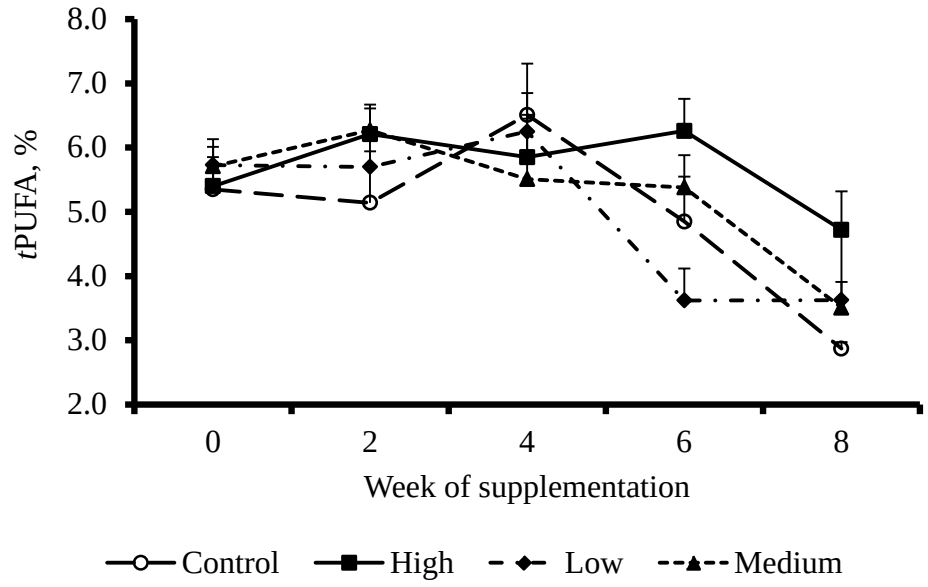

Figure 4.5 Weekly proportions of total polyunsaturated fatty acid (*t*PUFA) in milk of cows supplemented with varying levels of CDCO in the diet.

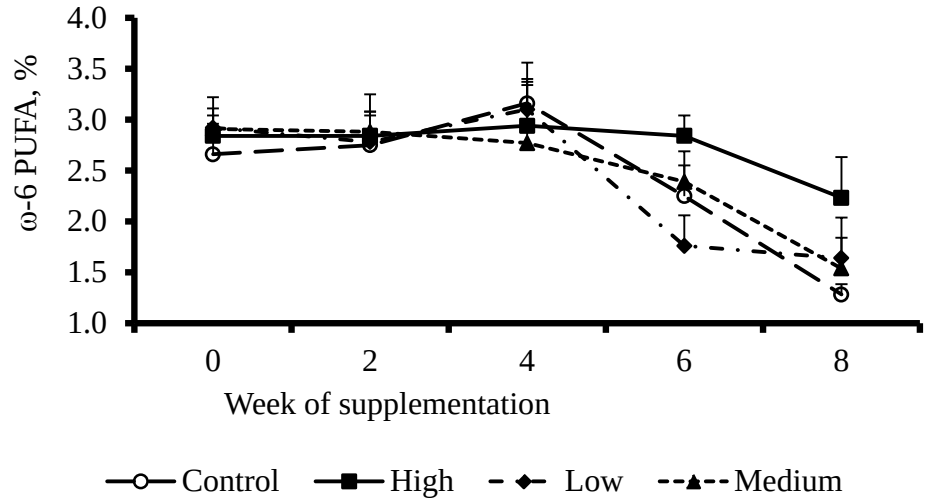

Figure 4.6 Weekly proportions of ω-6 fatty acids in milk of cows supplemented with varying levels of CDCO in the diet.

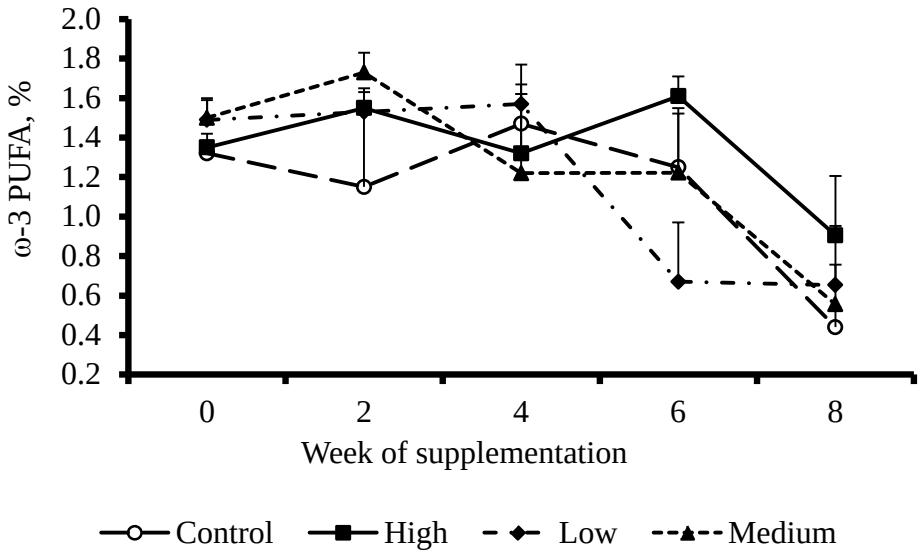

Figure 4.7 Weekly proportions of ω-3 fatty acids in milk of cows supplemented with varying levels of CDCO in the diet.

Discussion

The observed result in the current study where proportions of 18:1ω7t increased with incremental levels of CDCO is in agreement with previous studies in dairy cows that utilized canola seed, extruded linseed, and oils from rapeseed, soybean and canola as dietary supplements (Chichlowski *et al*., 2005; Jacobs *et al*., 2011; Kliem *et al*., 2011; Ferlay *et al*., 2013).The observed differences in the proportions of 18:1ω7t across treatment groups was possibly due to differences in the proportion of 18:1ω9c between the diets i.e. where the treatment diet (canola oil) had greater proportion of 18:1ω9c (41.90%) followed by the control diet (No canola oil: 16.50%) and lastly the basal diet (Pasture: 4.40%). This is in tandem with the findings of AbuGhazaleh et al. (2005), that enhancement of *trans*-18:1 fatty acid in milk is possible if a high concentration of dietary 18:1ω9c is available. In this current study, there were high 18:1ω9c proportions in the experimental diets that led to the observed variations in 18:1ω7t in the milk. Supplementation of lactating cows with CDCO had a positive impact on the proportion of 18:1ω9c in agreement with previous studies (Delbecchi *et al*. 2001; DePeters *et al*. 2001; Glasser *et al.,* 2008). Increased concentration of 18:1ω9c is usually associated with rumen biohydrogenation of 18:0, an essential precursor for the synthesis of 18:1ω9c (Banks, 1987; Chang *et al.,* 1992; Ntambi, 1999; Delbecchi *et al*., 2001; Hristov *et al*., 2011). It has also been reported that the majority of 18:1ω9c FA found in milk is as a result of desaturation of 18:0 fatty acids in the mammary gland (Enjalbert *et al*., 1998). Previous studies have also indicated that using rich vegetable sources of oleic acid is essential for enhancing the concentration of 18:1ω9c in milk fat (Bernard *et al*. 2005; Gómez-Cortés *et al*., 2008; Bodas *et al*., 2010).

The decrease in the concentration of *t*SFA is consistent with the known effect of canola/rapeseed on milk SFA profile (Aldrich *et al*., 1997; Glasser *et al.,* 2008). The productions of acetic, propionic, and butyric acids by rumen microbes as substrates for

energy synthesis, have been associated with the production in short and medium branched-chain SFA (Vlaeminck *et al*., 2006; Bernard *et al*., 2009). Therefore, the variation of *t*SFA between groups suggests that addition of CDCO in the diet of lactating cows possibly affected the activities of rumen microbes leading to milk fat depression. The proportion of milk *t*MUFA was high for cows in the high and medium CDCO treatment groups. This enhanced *t*MUFA is largely due to the elevated18:1ω9c in the diet (Delbecchi *et al*., 2001), which aligns with results of previous studies (Glasser *et al.,* 2008; Ferlay *et al*., 2013). The increasing level of *t*MUFA at the expense of *t*SFA observed in the current study could be beneficial to human health (Williams, 2000). No significant treatment differences were observed in the proportions of *t*PUFA, ω-3 and ω-6 fatty acids (Table 4.3), while 18:1ω7t, 18:1ω9c and *t*PUFA were significantly influenced by the duration (week) of CDCO supplementation (Table 4.4). This lends credence to the report of Martínez Marín et al. (2013) who demonstrated that in goats, time is an important factor in the modification of milk fatty acids. Therefore, the duration of supplementation may be just as crucial as the dietary composition in modifying milk FA composition in grazing cows. Therefore, the current results seem to suggest that to enhance the proportions of 18:1ω9c, 18:1ω7t and *t*MUFA at the expense of *t*SFA, primiparous Holstein-Friesian dairy cows grazing pastures need to be supplemented with CDCO at levels greater than 35 ml/kgDM for duration of eight weeks and *t*MUFA will continue to rise linearly at the expense of *t*SFA. Our current study also did agree with the findings of Ferlay et al. (2013) who found that feeding linseed to dairy cows increased the proportion of MUFA at the expense of SFA.

Implication of results on reproductive biomarkers

The present study has shown the potential of CDCO to increase the levels of essential fatty acids reaching key tissues of dairy cows. Essential LC-PUFA are known to impact positively on reproductive hormones (Gulliver et al., 2012). Previous studies have examined the effect

of fat supplementation on reproductive traits of ruminants (Sturmey et al., 2009; Hess et al., 2008). However, most of these studies focussed on the effect of total dietary lipid and energy balance, without investigating the effect of specific fatty acids (ω-6 and ω-3) on reproductive biomarkers (Santos et al., 2008; Funston, 2004; Staples et al., 1998). It was previously established that ω-6 and ω-3 have the potential to improve fertility traits and reproductive biomarkers (PGF2α, P4 and E2; Abayasekara & Wathes, 1999). Omega-3 has been implicated in the reduction of PGF2α synthesis in the endometrium (Mattos et al., 2004), whereas E2 and P4 concentrations in dairy cows were found to increase (Caldari-Torres et al., 2006; Zachut et al., 2011).

PUFA and steroidogenesis

CDCO significantly increased the concentration of *t*MUFA in dairy cow milk. The concentration of *t*PUFA significantly increased over time. The results of the current study indicate that CDCO can be used to increase the concentration of steroid hormones of pasture-based cows. The important roles that P4 and E2 play in dairy reproduction are well known and reductions in the concentration of either hormone can be detrimental to reproductive performance postpartum (Walsh et al., 2011). Previous studies have demonstrated that inclusion of dietary fat sources containing PUFA to the ration of dairy cows can improve the availability of P4 and E2 in granulosa cells and CL (Zachut et al., 2011; Robinson et al., 2002). A diet high in ω-3 reduces the availability of cholesterol, but because ω-3 also reduces the synthesis of PGF2α, it is thought that it stimulates a sustained release of cholesterol for the synthesis of steroid hormones (Santos et al., 2008). Conversely, rich sources of ω-6 are highly associated with the production of cholesterol. Cholesterol stimulates the expression of steroidogenic acute regulatory (StAR) protein, which stimulates the synthesis of P4 (Niswender, 2002). Cholesterol transported into the inner cells from the cytoplasm is converted to pregnenolone in the presence of Cytochrome P450 side chain cleavage enzyme

and then to P4 by 3β-hydroxysteroid dehydrogenase/Δ5, Δ4 isomerase (3β-HSD; Niswender, 2002). Polyunsaturated fatty acids are strongly linked with the increased expression of PPARs (MacLaren et al., 2006).

Conclusions

The observed increases in the proportions of 18:1ω9c, 18:1ω7t and *t*MUFA at the expense of *t*SFA suggest that the supplementation of grazing primiparous Holstein-Friesian cows with CDCO can potentially improve milk quality and enhance its beneficial healthy FA profile without any negative impact on the animals or milk taste. The present study has also shown the potential of CDCO to increase the levels of essential fatty acids reaching key productive and reproductive tissues of dairy cows. Therefore, the tested hypothesis that incremental supplementation of grazing primiparous Holstein-Friesian cows with CDCO will alter milk fatty acid composition towards increased total monounsaturates holds true and should be accepted.

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Chapter 5 : Effect of crude degummed canola oil and *ad libitum* grazing on plasma metabolites of primiparous Holstein-Friesian cows in a pasture-based system

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Abstract

The objective of this study was to investigate the changes in plasma metabolite profiles of pasture-based, primiparous, Holstein-Friesian cows supplemented with varying dietary levels of CDCO for eight weeks. The study tested the hypothesis that feeding grazing primiparous Holstein-Friesian cows for eight weeks with incremental levels of CDCO supplement will decrease plasma NEFA and BHBA, but increase plasma cholesterol and glucose metabolites. Twenty lactating primiparous Holstein-Friesian cows 40 days in milk were randomly allotted into four treatment groups that consisted of a wheat-based, pelleted basal diet with no supplemental CDCO (control), basal diet with CDCO added at 25 ml/kgDM (low), 35 ml/kgDM (medium) and 50 ml/kgDM (high) in an eight-week feeding trial, after two weeks of adjustment. Treatment influenced BHBA but had no effect on plasma NEFA, cholesterol and glucose metabolite profiles ($P>0.05$). However, week of supplementation had a significant effect ($P < 0.05$) on BHBA, NEFA and glucose concentrations. We concluded that with the exception of BHBA, CDCO at current levels of supplementation does not alter the plasma metabolite profiles of grazing primiparous cows. The lack of significant differences across treatments seems to indicate that higher levels of CDCO than the current levels used in this study, are probably needed. Furthermore, the duration of supplementation with CDCO had a greater impact on plasma metabolites than the levels of supplementation. Our findings also suggest that primiparous cows grazing high quality pastures during spring have sufficient energy intakes to prevent negative energy balance at 40 days in milk without the need for added fat supplements.

Keywords: Primiparous Holstein-Friesians, Crude degummed canola oil, Supplement, Plasma metabolites

Introduction

The supplementation of fat to lactating dairy cows has long been used as a management tool to increase dietary energy density for improving cow production, reproduction and to alleviate NEBAL (Beam & Butler, 1998; De Vries & Veerkamp, 2000). Attempts have been made to investigate the effect of canola meal on plasma metabolites in lactating cows, but the results have been diverse and inconsistent. To our current knowledge, there is a dearth of published information on the utilization of CDCO in pasture-based dairy systems. Primiparous Holstein-Friesian cows are the most energy challenged animals on a typical pasture-based dairy farm, because in such a herd, they are at the bottom of the social hierarchy (Moran & McLean, 2001). This is because primiparous cows are always the last to be milked and by implication, arrive last in the paddock, thus potentially reducing grazing time. This pattern, coupled with incidences of bullying and competition for grass in the paddock, contributes to low feed intake. Given that most first-time calvers are still heifers that are not fully grown at the time of calving (85-90% of mature cow size), they have to regain post-partum weight loss (up to 100 kg of pre-calving weight) and also continue to grow and produce milk (Moran & McLean, 2001). Therefore, primiparous cows tend to suffer more NEBAL than all the animals in the herd. With all these pressures and mating occurring fairly soon after calving, it is no wonder that they tend to have diminished milk production and reproduction performances.

Canola plant has been engineered to produce oil with greater concentrations of ω-9, ω-6 and adequate ω-3 FA. Metabolism of canola oil in the rumen is facilitated by rumen microorganisms, particularly bacteria and protozoa. Bacterial lipase hydrolyses the triacylglycerols and phospholipids in the consumed dietary oil. Once the fatty acids are liberated from their ester linkages, the end products (glycerol and NEFA) are utilised in the biohydrogenation process. Biohydrogenation is an extensive microbial process that involves

the addition of hydrogen molecules to unsaturated free fatty acids concentrated in the rumen. During biohydrogenation, unsaturated fatty acids (ALA and LA) are extensively hydrogenated to form saturated fatty acids (stearic acid 18:0 and palmitic acid 16:0). The biohydrogenation of linoleic acid to stearic acid is demonstrated in Figure 4.1 (Bauman & Griinari, 2001). Following biohydrogenation, the saturated and unsaturated fatty acids that escape this process are subsequently absorbed in the small intestine. As a result of rumen biohydrogenation, approximately 85% and 15% saturated and free fatty acids respectively, are transported into the small intestine and this process illustrates the efficiency of rumen microbes. Rumen biohydrogenation is the major factor affecting the delivery of fat in the small intestine and subsequent transportation in the blood of ruminants. Fat consumption by cows can lead to the productions of total volatile fatty acids and molar proportion of propionate (Onetti *et al.*, 2001; Li *et al.*, 2015). These volatile fatty acids are the precursors for the production of glucose, carbohydrates and fats.

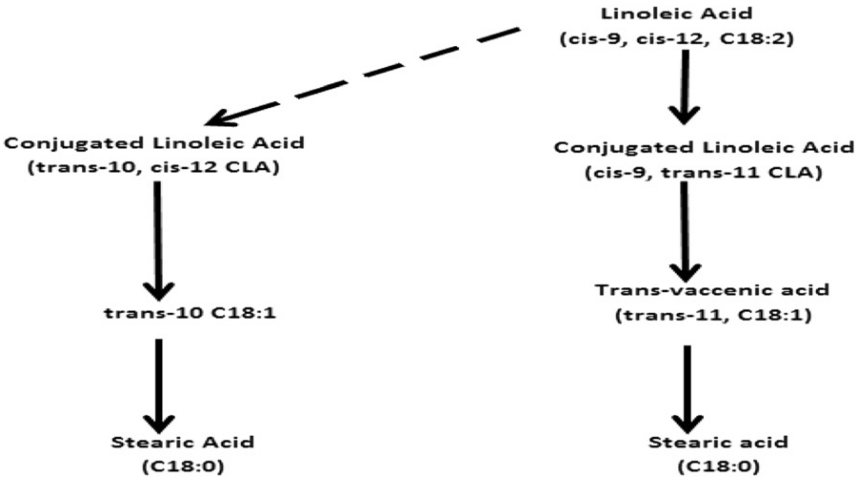

Figure 5.1 Biohydrogenation of linoleic acid to stearic acid.

The energy status of a cow is mostly reflected by its plasma NEFA and BHBA profiles (Grummer & Carroll, 1991; Butler, 2000, 2003; Leroy *et al*., 2005; Colazo *et al*., 2009; Lopes *et al*., 2011). A reduction in the level of plasma glucose can also be used as an indicator of

NEBAL in cows. Previous studies have suggested that NEFA concentration should be less than 0.2 mmol for normal cows (Drackley *et al.,* 2001). However, values ranging from 0.5 to 0.7 mmol postpartum are indicative of NEBAL (Adewuyi *et al.,* 2005). To prevent NEBAL in lactating cows, supplementation with limited amount of dietary fat in a pasture setting to boost postpartum energy intake has been of increasing research interest (Schroeder *et al*., 2004). The effect of dietary fat supplements on plasma metabolites in dairy cows has been inconsistent and highly variable in the published literature. For instance, some studies found increased glucose, NEFA, BHBA and cholesterol (Khorasani *et al*., 1992; Khorasani *et al.,* 1998; Delbecchi *et al*., 2001; Hayirli *et al.,* 2011), while others found no change (Bellows *et al*., 2001; Bottger *et al*., 2002; Chelikani *et al*., 2004; Chichlowski *et al*., 2005) or had inconsistent results (LaCount *et al*., 1994). This suggests that further studies in different production systems are required to enable informed choices and tailored decisions when feeding lactating cows with specific dietary fat supplements, hence the justification for our study in a typical Australian pasture-based dairy production system.

The Australian dairy industry has increasing interest in CDCO because of its ease of local availability and affordability. However, limited information currently exists in the published literature on the effect of CDCO on plasma metabolites. Therefore, this study intends to fill this knowledge gap by investigating the effect of dietary inclusion of CDCO at incremental levels for eight weeks on the plasma metabolite profiles of primiparous Holstein-Friesian cows in a pasture-based system. We hypothesized that feeding grazing primiparous Holstein-Friesian cows for eight weeks with incremental levels of CDCO supplement will decrease plasma NEFA and BHBA, but increase cholesterol and glucose levels.

Materials and methods

Site and climatic conditions

All experimental procedures were in accordance with the University of Tasmania Animal Ethics Committee guidelines (Animal Ethics Permit Number A0012583), the 1993 Tasmania Animal Welfare Act and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The experiment was carried out at the University of Tasmania's Dairy Research Centre, Tasmanian Institute of Agriculture (TIA) Elliot Dairy Research Farm in Somerset, North-Western Tasmania, Australia, from September to November 2012. Tasmania is Australia's smallest state with a land size of 68,000 square kilometres and located within the cool, temperate, climatic zone at latitude 42° South and longitude 147° East. It is characterized by four distinct seasons; winter, autumn, spring and summer. The experiment was carried out in spring when the annual rainfall was 2500 mm and humidity was approximately 60%.

Animals and Treatments

The condition and energy status of the experimental cows were visually assessed based on BCS on a scale of 1–8 (DPI, 2003; Stockdale, 2001). Twenty primiparous, spring-calving, purebred, Holstein-Friesian cows (average liveweight of 400 ± 40 kg, BCS 4 ± 1, 40 ± 8 DIM early lactation phase; and daily milk yield of 20.7 litres), were randomly allocated into 1 of 4 treatments of CDCO (25 ml/kgDM, 35 ml/kgDM and 50 ml/kgDM) and the control (no CDCO- 0 ml/kgDM). For the supplementation trial, a complete randomise experimental design (CRD) was applied. This replicated herd of cows (n = 5 per treatment group) receiving CDCO supplements was placed under the same management and rotated in electric fenced paddocks with the control cows offered wheat-based pellets without CDCO. Together, the animals had access to 3000 $kgDMha^{-1}$ of forages, a mixture of ryegrass (*Lolium perenne*), cocksfoot (*Dactylis glomerata*), and white clover (*Trifolium repens*) pasture grazed at the

two-leaf stage. Water was offered at *ad libitum*. The current level of CDCO was calculated based on 7% total fat recommended for grazing cows (Schroeder *et al*., 2004) and the physiological status of being in the early lactation phase. Each cow received 6 kg of the pelleted supplements daily for eight weeks, after two weeks of adjustment. Supplements were offered to cows in two splits; morning (3 kg) and evening (3 kg) milking sessions at 05:00 h and 15:00 h. There were no orts from any of the groups. The exact pasture intake was difficult to estimate as the case is under grazing conditions.

Feed chemical composition and analysis

Dry matter (DM) content of the basal and experimental diets was determined by drying samples to a constant temperature at 65°C in a fan forced oven, finely ground to pass through a 2 mm sieve using Laboratory Mill (Thomas Model 4 Wiley® Mill; Thomas Scientific), and further drying at 105°C for 24 h. The DM was computed as the difference between the initial and final weights of samples expressed as a percentage. Moisture content was determined by subtracting the % DM from 100%. Ash content was determined by combusting samples in a furnace at 600°C for 8 hours. NDF and ADF contents were measured using an Ankom fiber analyser, ANKOM220; ANKOM Technology, USA. The analysis for total nitrogen was determined using a Thermo Finnigan EA 1112 Series Flash Elemental Analyser and the values multiplied by 6.25 to give the CP percentage. Ether extract (EE) was determined using an Ankom fat/oil extractor (ANKOMXT15; ANKOM Technology, USA) based on hexane-petroleum ether solvent extraction. Metabolisable energy was calculated as per Van Es (1975). The chemical compositions of the treatment, control and basal feeds are presented in Table 5.1.

Table 5.1 Chemical composition of the experimental, control and basal feeds.

Chemical Composition (%DM)	Feeds		
	Control (No canola oil)	Treatment (canola oil)	Basal diet (Pasture)
MC	9.1	8.2	5.5
DM	90.9	91.8	94.5
ADF	9.0	8.0	27.7
NDF	21.1	20.0	45.9
EE	2.1	6.2	3.0
Ash	8.9	9.7	9.3
NFC	59.0	52.8	23.9
OM	91.1	90.3	90.7
CP	10.4	12.7	21.0
ME (MJ/kg DM)	4.07	4.08	3.99

All feeds were analysed based on a dry weight basis; Moisture content (MC), Dry matter (DM), organic matter (OM), neutral detergent fibre (NDF), acid detergent fibre (ADF), non-fibrous carbohydrate (NFC), ether extract (EE), crude protein (CP) and Metabolisable energy (ME). Treatment = feed with added canola oil. Control = feed without canola oil, Basal diet = mainly mixed ryegrass pasture.

Blood sample collection and plasma metabolite analysis

Blood samples (10 ml) were collected from each experimental cow after the morning milking (05:00 h) on week zero and fortnightly thereafter, until the end of the experiment. All samples were collected by coccygeal venepuncture into heparin vacutainers. All collected blood samples were centrifuged at 1,125 X g for 10 minutes at 4°C to facilitate distinct plasma separation. The plasma fractions were decanted into 2 ml vials, sealed with an airtight cap and stored at −20°C until further laboratory analyses. Plasma NEFA, BHBA, cholesterol and glucose samples were analysed at the Animal Health Laboratories, Department of Agriculture and Food (South Perth, Australia) using appropriate kits (ACS-ACOD Method) from Wako Pure Chemical Industries Ltd (Code No. 279–75401) on Beckman Coulter (AU 400) analyser.

Statistical analysis

Initially, summary statistics by level and week of CDCO supplementation were computed to give means, standard deviations standard error, variance, minimum and maximum values that were scrutinised for any data entry errors. Testing for linear, quadratic and cubic orthogonal contrasts by regressing the dependent on explanatory variables was carried out using PROC REG (SAS, 2009). Subsequently, NEFA, BHBA, glucose and cholesterol were analysed by repeated measures analysis of variance using PROC MIXED (SAS, 2009) utilising 1^{st}-order autoregressive covariance structure. 1^{st}-order autoregressive covariance structure was utilised because it has homogeneous variances and correlations that decline exponentially with distance i.e. variability in measurement is constant regardless of when you measure it. Week of supplementation fitted as the repeated effect. The model included treatment, week of lactation and interaction between treatment and week of lactation as fixed effects, while base line metabolite values fitted as covariate and cows were fitted as random effect and the degrees of freedom were estimated by the Satterthwaite method (SAS, 2009). Variables of interest having significant treatment and or week of lactation effects are presented in Tables and Figures as Least Squares Means and Standard Error (LSM ± SEM) and differences between means were considered significant at the $P < 0.05$ threshold unless otherwise stated. Significant differences and mean separations were carried out using Tukey's probability pairwise comparison tests (SAS, 2009). Pearson correlation coefficients between dependent variables were estimated using PROC CORR (SAS, 2009) with significance determined using Bonferroni's probability pairwise comparison test (SAS, 2009). Correlation analyses were initially carried out on the whole data set and also by week of supplementation, but the weekly correlation values were dropped and those from the whole data retained because there were no significant differences between the two sets of values.

Results

Effect of CDCO level and week of supplementation on plasma metabolites

Crude degummed canola oil supplementation level did not significantly (P>0.05) affect plasma NEFA. However, week of supplementation had a highly significant effect (P < 0.05) on plasma NEFA. Also, no significant interaction effect of treatment by week was detected on plasma NEFA (P > 0.05; Table 5.2 and 5.3).

Table 5.2 The least square means (LSM ± SEM) of plasma metabolites of primiparous Holstein Friesian as influenced by CDCO treatment and week of supplementation.

Effect	Plasma metabolites (mmol)			
	BHBA	Cholesterol	Glucose	NEFA
Control	0.5±0.0^{a}	5.8±0.2	3.9±0.1	0.2±0.0
Low	0.4±0.0^{b}	5.3±0.3	3.7±0.2	0.1±0.0
Medium	0.4±0.0^{b}	5.5±0.2	3.9±0.1	0.2±0.0
High	0.5±0.0^{a}	5.6±0.3	3.9±0.1	0.2±0.0
Week				
0	0.4±0.0^{b}	5.5±0.2	4.1±0.1^{a}	0.2±0.0^{a}
2	0.6±0.0^{a}	5.6±0.2	4.0±0.1^{ab}	0.1±0.0^{b}
3	0.4±0.0^{b}	5.6±0.3	3.8±0.1^{ab}	0.2±0.0^{a}
5	0.6±0.0^{a}	5.5±0.3	4.0±0.1^{ab}	0.1±0.0^{b}
7	0.4±0.0^{b}	5.3±0.5	3.5±0.2^{c}	0.2±0.0^{a}
8	0.4±0.0^{b}	5.7±0.4	3.7±0.1^{bc}	0.1±0.0^{b}

Column means within a variable bearing different superscripts significantly differ (P<0.05); β-hydroxybutyrate (BHBA), non-esterified fatty acid (NEFA), Cholesterol, Glucose; Crude degummed canola oil (CDCO). Each group had five cows. Week 0, week before supplementation, week 2, second week of fat supplementation.

Table 5.3 The fixed and interaction effects (p-values) of CDCO treatment and week of supplementation on plasma metabolites of primiparous Holstein Friesian.

Plasma Metabolites (mmol)	P values		
	TRT	Week	TRT*Week
BHBA	**0.0241***	**0.0001*****	0.6489^{NS}
Cholesterol	0.6681^{NS}	0.9415^{NS}	0.9962^{NS}
Glucose	0.4143^{NS}	**0.0005****	0.2613^{NS}
NEFA	0.1314^{NS}	**0.0001*****	0.8714^{NS}

All P-values in bold were significant (P<0.05). Level of significance: * significant (P<0.05), ** highly significant (P<0.01), *** very highly significant (P<0.001), ^{NS} not significant (P>0.05); β-hydroxybutyrate (BHBA), non-esterified fatty acid (NEFA), Cholesterol, Glucose; Crude degummed canola oil (CDCO).

Plasma BHBA was significantly affected by both treatment (P<0.05) and week of supplementation (P<0.05; Table 5.2), although their interaction (treatment by week), was not significant (P>0.05; Table 5.3). However, plasma BHBA concentration in cows receiving the high treatment was similar to that of cows in the control group (0.5 ± 0.0 vs 0.5 ± 0.0 mmol), but differed (P<0.05) from those of cows receiving low (0.4 ± 0.0 mmol) and medium (0.4 ± 0.0 mmol) levels of CDCO (Table 5.2). There were no significant (P>0.05) differences in the mean plasma cholesterol and glucose concentrations of supplemented and unsupplemented cows. However, week of supplementation influenced glucose significantly (P<0.05) as the level fell down in week seven (Table 5.2), but cholesterol was not affected (P>0.05; Table 5.3).

Correlations between traits

Table 5.4 shows that there were highly significant correlations (P<0.001) between BHBA and NEFA (−0.32), cholesterol (0.24) and glucose (0.34). All other correlations were not significant (P>0.05).

Table 5.4 Pearson's correlation coefficients between plasma metabolites

Traits	BHBA	Cholesterol	Glucose	NEFA
BHB(mmol)		0.24*	0.34***	-0.32***
Cholesterol(mmol)	0.24*		0.25**	-0.02^{NS}
Glucose(mmol)	0.34***	0.25**		0.03^{NS}
NEFA(mmol)	-0.32***	-0.02^{NS}	0.03^{NS}	

Level of significance: ^{NS} not significant (P>0.05), * significant (P<0.05), ** highly significant (P<0.01), *** very highly significant (P<0.001); β-hydroxybutyrate (BHBA), non-esterified fatty acid (NEFA).

Weekly trends in plasma metabolites of supplemented and unsupplemented cows

The weekly concentration trends of NEFA (Figure 5.2), cholesterol (Figure 5.4) and glucose (Figure 5.5) were similar across treatments and the control groups. However, the weekly BHBA trends for cows in the high group were higher compared to the medium, low and control groups (Figure 5.3).

There were significant drops in the levels of plasma NEFA in week two, five and eight for all the treatment groups (Figure 5.2). In week three and seven, the high group produced the highest level of plasma NEFA, followed closely by the control groups (Figure 5.2). Generally however, in week three and seven, most groups seem to have elevated levels of plasma NEFA (Figure 5.2). The concentration of plasma BHBA peaked in week two, before a drastic fall in week three and, a rapid incline in week five before tapering off in weeks seven and eight (Figure 5.3).. The control group produced consistent level of plasma BHBA from week two through eight (Figure 5.3). The Low and the medium groups both produced the lowest level of plasma levels in weeks three, five and seven (Figure 5.3). The low group consistently produced low level of plasma cholesterol from week two through seven before a rapid surge

to a peak in week eight (Figure 5.4). However, the control, medium and high groups produced similar concentrations of cholesterol in plasma (Figure 5.4). The control and the low groups yielded the lowest levels of plasma glucose in weeks two and seven, respectively (Figure 5.5).

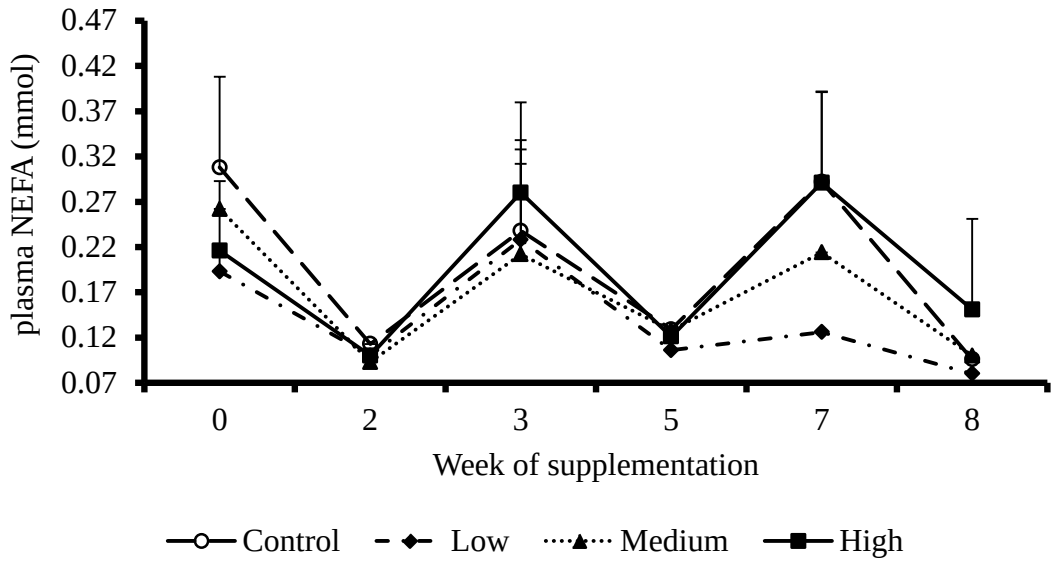

Figure 5.2 Weekly plasma concentrations of non-esterified fatty acids of cows supplemented with varying levels of CDCO in the diet. Error bars (±SEM). Each treatment group had five cows. Week 0, week before fat supplementation, week 1, when fat supplementation commenced.

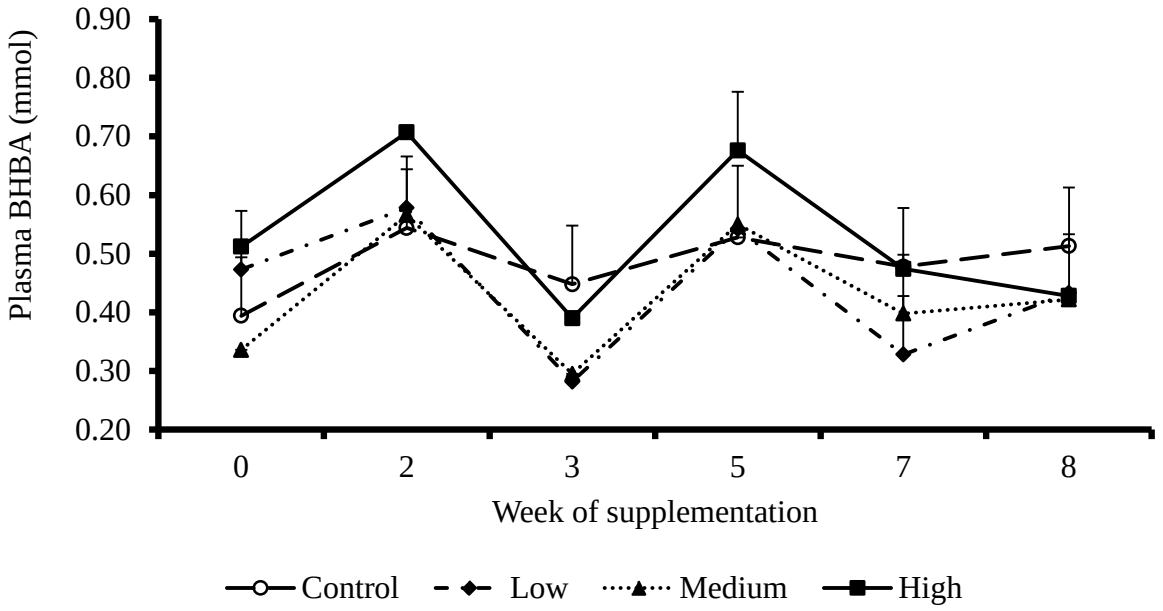

Figure 5.3 Weekly plasma concentrations of β-hydroxybutyrate of cows supplemented with varying levels of CDCO in the diet. Error bars (±SEM). Each treatment group had five cows. Week 0, week before fat supplementation, week 1, when fat supplementation commenced.

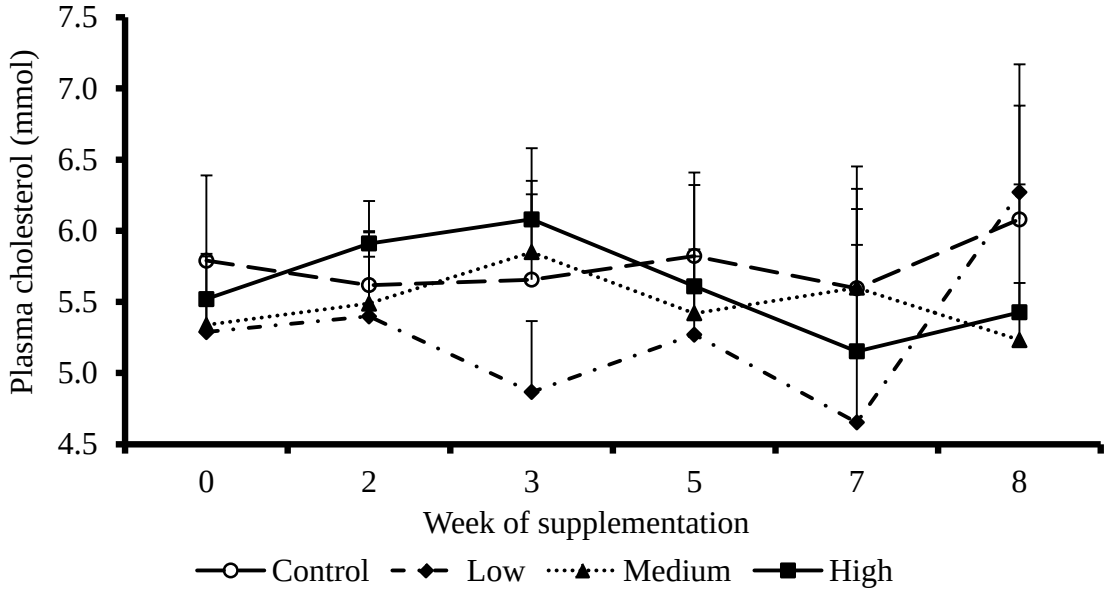

Figure 5.4 Weekly plasma concentrations of cholesterol of cows supplemented with varying levels of CDCO in the diet. Error bars (±SEM). Each treatment group had five cows. Week 0, week before fat supplementation, week 1, when fat supplementation commenced.

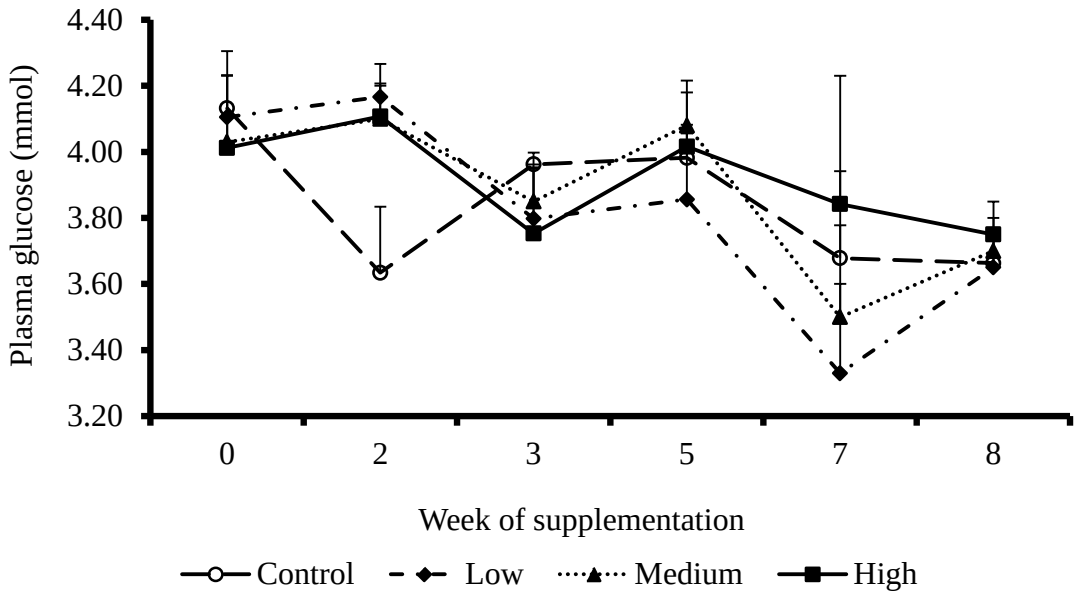

Figure 5.5 Weekly plasma concentrations of glucose of cows supplemented with varying levels of CDCO in the diet. Error bars (±SEM). Each treatment group had five cows. Week 0, week before fat supplementation, week 1, when fat supplementation commenced.

Discussion

The results of our current study found no significant effect of feeding CDCO on NEFA concentration in contrast to Douglas et al. (2007) and Andersen et al. (2008) who reported increases in NEFA. These previous studies utilized different forms and higher dosages of dietary fat sources which could explain the observed differences. The CDCO levels utilised in our study were tailored to avoid feeding greater than the 7% total fat recommended in the diets of grazing animals (Schroeder *et al*., 2004) because it can affect dry matter intake and ruminal fibre digestion (Schroeder *et al*., 2004). Another explanation for the lack of observable differences between the supplemented and unsupplemented groups of animals in our study could be related to the metabolisable energy component of the CDCO which was very similar to those for the control and basal diets (isocaloric). Secondly, the result also suggests that the ryegrass pastures that were grazed by the cows were of high quality and provided adequate energy to the postpartum cows to prevent massive adipose tissue remobilisation. The similarity across weeks and between treatment groups (average 0.2 mmol) in plasma NEFA suggests similar energy intake and utilisation. It has been suggested that three weeks pre-partum to three weeks post-partum is the most energy deficit period for high merit cows (Drackley, 1999), but the cows in our study were already 40 DIM (days in milk) and might have well passed the critical energy deficit periods, hence the lack of observable treatment differences. It has also been reported that the level of plasma NEFA was greater in primiparous cows soon after calving (Wathes *et al*., 2007), implying that fat supplementation studies on NEFA should be carried out during the early calving period (Meikle *et al*., 2004).

Plasma BHBA is a product of NEFA that is converted into triacylglycerols in the liver (Heitmann & Fernandez, 1986; Roche *et al*., 2008). In the present study, feeding CDCO significantly influenced plasma BHBA concentration where a slight, but significant increase

in BHBA was observed as the level of CDCO increased from low to high (Table 5.2). Our results are in contrast with those of (Duske *et al*., 2009) who found that when rumen inert fat was fed to dairy cows, plasma BHBA decreased prepartum. This disparity could be linked to the differences in the physiological states of the experimental cows. In our study, the cows were only 40 DIM coinciding with the early lactation phase, while in their study, the cows were 300 days in milk almost at the end of their lactation. Another major area of difference between the two studies lies in the type of fat fed. While we supplemented the cows with crude degummed canola, they fed an inert fat that is rumen-protected. The effect of fat supplementation on plasma BHBA has been associated with the impact of long chain fatty acids (>C18:0: particularly Docosahexaenoic acid, C22:6, C18:1 and C18:3) on hepatic gluconeogenesis (Mashek & Grummer, 2003). It would thus appear that in our study, as the level of CDCO supplementation increased, hepatic gluconeogenesis also increased due to low level of C22:6, and adequate levels of C18:1 and C18:3 in the treatment diet.

Cholesterol contains lipoproteins and the high and low density lipoproteins (Grummer & Carroll, 1988; Bauchart, 1993; Staples *et al.,* 1998), which are all precursors for progesterone synthesis (Staples *et al.,* 1998). Progesterone is one of the hormones essential for fertility in cows. Fat supplements have been used efficiently to alter the plasma cholesterol concentration of dairy cows (Carroll *et al*., 1990; Hawkins *et al*., 1995). However, in the current study, dietary fat did not influence plasma cholesterol concentration. This can partly be explained by the fact that canola contains phytosterol (Hamama *et al.,* 2003; Vlahakis & Hazebroek, 2000). Previous reports have shown that phytosterol contains low levels of cholesterol (Hamama *et al.,* 2003). Previous studies have also reported that phytosterol can significantly reduce cholesterol in humans with hypercholesterolemic condition (Miettinen *et al*., 1995). Therefore, the lack of significant effect observed in the present study could be explained by the hypocholesterolemic effect of CDCO.

The demand for glucose rises sharply post-partum due to increased energy requirements for lactation (Drackley *et al.*, 2001). However, due to low dry matter intake after parturition, the amount of glucose produced is not enough to support the cow's lactation requirements. Ruminal propionate produced during ruminal fermentation is used as a substrate in the gluconeogenesis pathway to produce glucose (Williams & Stanko, 1999; Howlett *et al*., 2003; Funston, 2004). The effect of dietary fat supplements on plasma glucose has been associated with their ability to enhance adequate rumen propionate (Williams & Stanko, 1999; Howlett *et al*., 2003). In the current study, there were no significant differences between CDCO supplemented and unsupplemented cows. The lack of significance in the present result indicates that the canola oil-supplemented and unsupplemented groups both enhanced adequate propionate production in the rumen to affect plasma glucose concentration. In addition, the effect of biohydrogenation of unprotected supplementary fat in the rumen was not different between supplemented and control group of cows, hence the availability and levels of substrates necessary for glucose metabolism were similar in all cows. In most previous reports, unprotected fats are mostly subjected to rigorous rumen biohydrogenation than the protected fat, hence different outcomes (Jenkins, 1993; Chilliard *et al*., 2007).

Week of supplementation had a significant influence on NEFA, BHBA and glucose. This seems to suggest that the longer cows are supplemented with CDCO, the greater the impact on plasma metabolite profiles. The negative correlation between NEFA and cholesterol, and the positive correlation between cholesterol and glucose in the present study corroborate the theory that negative energy balance can impact negatively on reproduction parameters in a pasture based setting.

When plasma glucose levels decrease, body fat remobilization is instigated from nutrient accrual to provide sufficient energy that can maintain continuous milk production until the

animal returns to a positive energy balance. It has been found that cows suffering from negative energy balance have increased concentrations of serum glucagon and growth hormones, whereas the concentrations of insulin and insulin growth factor-I are decreased. Some proposed theories postulate that dietary fat supplement favours lower blood NEFA concentration by providing extra energy postpartum. Other research findings (Blum et al., 1985; Baumgard et al., 2000; Lucy & Crooker, 2001) indicate that feeding dairy cows with fat supplements could promote increased insulin production because of the amount of increased energy provided through the production of propionate, a precursor for glucose production. However, studies investigating the response of plasma insulin to fat supplementation are inconsistent. For instance, some studies reported decreased plasma concentrations of insulin, while others reported steady insulin increases postpartum in cows fed six different diets containing fats (Lucy *et al.,* 1991). Therefore the mechanism of fat supplementation and relationship with insulin production is still poorly understood and warrants further elucidation.

Conclusions

Canola supplements are effective dietary energy sources because in the rumen they produce fatty acids which serve as lipid metabolic substrates for the synthesis of glucose and fats for lactation. The concentrations of plasma NEFA, BHBA and glucose are indicators for gauging the energy balance status of a lactating cow. Week of supplementation was a more significant factor than level of CDCO supplementation in influencing plasma metabolite profiles, thus suggesting that the duration of supplementation with CDCO has a greater impact on all the plasma metabolites investigated in this study. It was also apparent from this study that primiparous cows grazing high quality pasture at about 40 DIM had adequate energy intake to overcome any extreme negative energy balance scenario at this stage of lactation. It also implies that fat supplementation may not be necessary in spring when there is abundant and

lush pasture, but may be needed during winter or summer when pasture is scanty to boost the energy intake of cows. The hypothesis that feeding grazing primiparous Holstein-Friesian cows for eight weeks with incremental levels of CDCO supplement will decrease plasma NEFA and BHBA, but increase plasma cholesterol and glucose metabolites does not hold true and should be rejected. Therefore, it is concluded that primiparous Holstein-Friesian dairy cows in a pasture-based setting have enough energy intakes from grass in spring to maintain adequate production and reproduction performances. However, there is the need for further investigation into the interaction between circulating plasma hormones and gene expression profiles of supplemented cows to provide a better understanding of CDCO's role in future applications as a dietary fat supplement for lactating cows.

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Chapter 6 : Effect of incremental levels of crude degummed canola oil on milk progesterone, plasma luteinizing and follicle stimulating hormones of primiparous Holstein- Friesian cows in a pasture-based system

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Abstract

Dietary supplementation of lactating cows with fat can alter the profiles of key reproductive hormones and boost postpartum energy balance. However, published data under Australian pasture-based dairy production conditions are scanty and inconsistent. Therefore, the objective of this study was to determine whether dietary inclusion of CDCO at incremental levels for eight-weeks will have significant influence on P4, LH and FSH of primiparous Holstein-Friesian cows grazing pastures. We tested the hypothesis that postpartum supplementation of primiparous Holstein-Friesian cows with dietary CDCO in a pasture-based system will alter the concentrations of P4, LH and FSH reproductive hormones. A random allocation of twenty primiparous Holstein-Friesian cows into four treatment groups that consisted of a wheat-based pelleted basal diet with no supplemental CDCO (control), or a wheat-based pelleted basal diet with CDCO added at 25 ml/kgDM (low), 35 ml/kgDM (medium) and 50 ml/kgDM (high) was employed in an eight-week feeding trial after two weeks of adjustment. Supplementation levels of CDCO and week of data collection were significant sources of variation ($P<0.05$) that influenced FSH and P4 concentrations. However, there was no significant effect of supplementation on LH concentration ($P>0.05$). It was apparent that cows in the high (0.459 ng/ml), medium (0.367 ng/ml) and low (0.251 ng/ml) levels of oil treatments had higher mean plasma FSH concentrations compared to the control (0.172 ng/ml) cows. It was concluded that the current levels of CDCO can be used in pasture-based dairy systems to increase FSH, but not LH and P4.

Keywords: Crude degummed canola oil; Progesterone; Luteinizing hormone; Follicle stimulating hormone

Introduction

Primiparous Holstein-Friesian cows are known to have lower postpartum conception rates than multiparous cows in pasture-based systems (Moran & McLean, 2001). The pressures of milk production accompanied by NEBAL at this stage of lactation are the main reasons adduced for poor reproductive performance (Butler, 2003). The effect of NEBAL on oestrus cycle is well documented. Energy-challenged cows having cycles of poor reproductive performances have been associated with atypical reproductive hormonal profiles (Royal *et al*., 2000). It has been suggested previously that the secretion of steroids and gonadotropins is inhibited in cows suffering from NEBAL (Lucy *et al*., 1991; Royal *et al*., 2000). For instance, cows losing body condition were shown to have lower serum LH compared to cows gaining body condition postpartum (Rutter & Randel, 1984; Randel, 1990). A study conducted in the UK revealed that pregnancy rate to first service declined from 55.6% to 39.7% in modern cows (Royal *et al*., 2000). Atypical hormonal pattern of the cows was implicated for the fertility decline (Royal *et al*., 2000). Another study conducted in Tasmania, Australia with over a million records of dairy cows from 428 pasture-based dairy farms, also revealed a decline in fertility (Malau-Aduli & Otto, 2013). Early resumption of postpartum oestrus cycle is essential for reproductive performance in cows (De Fries *et al.,* 1998). However, it is dependent on the energy status and availability of adequate circulation of some key reproductive hormones; P4, LH and FSH in plasma (Grummer & Carroll, 1988; Forde *et al*., 2011).

Australian dairy farmers have utilized supplements (mainly wheat and barley) partially in seasons when rainfall is below average, to boost energy intake of individual cows to increase milk production. Fat supplementation is not popular in Tasmania because of its unknown effects on performances of cows. However, fat supplementation to dairy cows can provide two benefits; alteration of reproductive hormone patterns and increasing the energy density of

the rations consumed by lactating cows (Beam & Butler, 1997; Staples *et al.*, 1998; Royal *et al*., 2000). Studies conducted on the effect of fat supplementation on reproductive hormones in dairy cows have been conflicting and inconsistent (Carroll *et al.*, 1992; Staples *et al.*, 1998). For instance, some studies reported increased P4 concentrations in cows (Boken *et al*., 2005; Coyrall-Castel *et al.*, 2010; Caldari-Torres *et al*., 2011), while others either found no change (De Fries *et al.*, 1998) or a decrease in P4 concentrations (Robinson *et al*., 2002). Some studies have also shown that LH and FSH were influenced by fat supplementation, while in others, they were unaltered or decreased (Beam & Butler, 1997; Staples *et al.*, 1998). In addition, previous studies have mostly focused on grain-fed stall-barn dairy systems. There is limited published data on fat supplementation in pasture-based systems under Australian conditions. This suggests that further studies in different production systems are required to enable informed choices and tailored decisions when feeding lactating cows with specific dietary fat supplements, hence the justification for our study in a typical Australian pasture-based dairy production system. Therefore, we hypothesized that feeding crude CDCO to primiparous Holstein-Friesian cows for eight weeks in a pasture-based dairy system postpartum, could alter the concentrations of P4, LH and FSH reproductive hormones in milk and plasma. The objective of this study was to determine whether dietary inclusion of CDCO at incremental levels for eight weeks will have significant influence on the concentrations of P4, LH and FSH in primiparous Holstein-Friesian cows grazing pastures.

Materials and Methods

All experimental procedures were in accordance with the University of Tasmania Animal Ethics Committee guidelines, the 1993 Tasmania Animal Welfare Act and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Site and climatic conditions

The experiment was carried out at the University of Tasmania's Dairy Research Centre, Tasmanian Institute of Agriculture (TIA) Elliot Dairy Research Farm in Somerset, North-Western Tasmania, Australia, from September to November 2012. Tasmania is Australia's smallest state with a land size of 68,000 square kilometres and located within the cool, temperate, climatic zone at latitude 42˚ South and longitude 147˚ East. It is characterized by four distinct seasons - winter, autumn, spring and summer. The experiment was carried out in spring when the annual rainfall was 2500 mm and humidity was approximately 60%.

Animals and treatments

The condition and energy status of the experimental cows were visually assessed based on BCS on a scale of 1-8 (Stockdale, 2001; DPI, 2003). Twenty 20 primiparous, spring-calving, purebred, Holstein-Friesian cows (average liveweight of 400 ± 40 kg, BCS 4 ± 1 and 40 ± 8 days in milk (DIM), were randomly allocated into 1 of 4 treatments of the control (no CDCO-0 ml/kgDM), CDCO (25 ml/kgDM, 35 ml/kgDM and 50 ml/kgDM). For the supplementation trial, a complete randomise experimental design (CRD) was applied. This replicated herd of cows (n=5 per treatment group) receiving CDCO supplements was placed under the same management and rotated in electric fenced paddocks with the Control cows offered wheat-based pellets without CDCO. Together, the animals had access to 3000 $kgDMha^{-1}$ of forages, a mixture of ryegrass (*Lolium perenne*), cocksfoot (*Dactylis glomerata*), and white clover (*Trifolium repens)* pasture grazed at the two-leaf stage. Water was offered *ad libitum.* The treated cows grazed the same pasture allotment as the control cows but were offered CDCO

plus wheat-based pellet at the rate of 50 ml/kgDM (for the high level of supplementation group), 35 ml/kgDM (medium level of supplementation group) and 25 ml/kgDM (low level of supplementation group). The current level of CDCO was calculated based on 7% total fat recommended in the diet of grazing cows (Schroeder *et al*., 2004). Each cow received 6 kg of the pelleted supplements daily for eight weeks, after two weeks of adjustment. Supplements were offered to cows in two splits; morning (3 kg) and evening (3 kg) milking sessions at 05:00 h and 15:00 h. There were no orts from any of the groups. The exact pasture intake was difficult to estimate as the case is under grazing conditions.

Feed chemical composition and analysis

Dry matter (DM) content of the basal and experimental diets was determined by drying samples to a constant temperature at 65°C in a fan forced oven, finely ground to pass through a 2mm sieve using Laboratory Mill (Thomas Model 4 Wiley® Mill; Thomas Scientific), and further drying at 105^0C for 24 h. The DM was computed as the difference between the initial and final weights of samples. Ash content was determined by combusting samples in a furnace at 600°C for 8 hours. NDF and ADF contents were measured using an Ankom fibre analyser (ANKOM220; ANKOM Technology, USA). The analysis for total nitrogen was determined using a Thermo Finnigan EA 1112 Series Flash Elemental Analyzer and the values multiplied by 6.25 to give the CP percentage. Ether extract (EE) was determined using an Ankom fat/oil extractor (ANKOMXT15; ANKOM Technology, USA). Metabolisable energy was calculated as per Van Es (1975). The chemical compositions of the treatment, control and basal feeds are presented in Table 6.1.

Table 6.1 Chemical composition of the experimental, control and basal feeds.

Chemical composition (%DM)	Feeds		
	Control (No canola oil)	Treatment (canola oil)	Basal diet (Pasture)
MC	9.1	8.2	5.5
DM	90.9	91.8	94.5
ADF	9.0	8.0	27.7
NDF	21.1	20.0	45.9
EE	2.1	6.2	3.0
Ash	8.9	9.7	9.3
NFC	59.0	52.8	23.9
OM	91.1	90.3	90.7
CP	10.4	12.7	21.0
ME (MJ/kg DM)	4.07	4.08	3.99

All feeds were analysed based on a dry weight basis; Moisture content (MC), Dry matter (DM), organic matter (OM), neutral detergent fibre (NDF), acid detergent fibre (ADF), non-fibrous carbohydrate (NFC), ether extract (EE), crude protein (CP) and Metabolisable energy (ME). Treatment = feed with added canola oil. Control = feed without canola oil, Basal diet = mainly mixed ryegrass pasture.

Milk and blood sample collection

Milk samples (n=480) were collected 3 times a week during morning milking (05:00 h) throughout the 8 weeks experimental period to capture the ephemeral progesterone in milk. The milk sample collection was initiated before breeding (day -32) and completed after breeding (day 32). Aliquots of fresh milk samples were collected using MPC; 680 fitted onto the De Laval herringbone milking machine. The milk aliquots were stored in plastic vials at -20^{0}C until analysed. Blood samples were collected from each experimental cow after the morning milking (05:00 h) on the day before and after the initiation of the CDCO treatment, and weekly before and after commencement of oestrous cycle, this collection strategy was largely set to target the pulsatile nature of gonadotropins. However, the collection of blood samples was restricted by the guidelines of University of Tasmania Animal Ethics Committee, and so not enough blood samples could be collected to reflect the pulsatile nature of FSH and LH. All samples were withdrawn from the coccygeal vein into lithium heparin vacutainers. Approximately thirty minutes thereafter, all collected blood

samples were centrifuged using Eppendorf (5810 R) at 1,125 X g for 10 minutes at 4^0C. The plasma were harvested and then decanted into 2ml vials, sealed with an airtight cap and stored at -20^0C until analysed. Milk samples were analysed for milk P4 concentrations, while the plasma samples were analysed for LH and FSH concentrations.

Synchronization of the oestrus cycle

A double injection protocol over 14 days to synchronize oestrus was initiated in week three of dietary treatment with Ovuprost (2 ml), with artificial insemination on the morning of day 15. Ovuprost has a GnRH-PGF2a format. The Ovuprost was purchased from a local veterinary clinic (Wynyard, Tasmania, Australia) and injected by a trained technician. All cows had heat detector (ESTROTECT™) patches mounted on their caudal region after Ovuprost injection.

Progesterone assay

Milk P4 concentration was determined by competitive immunoassay, using appropriate P4 enzyme-linked immunosorbent assay (ELISA) kit (ENZO® Life Science, Lause, Switzerland), as described by (Tijssen, 1985). Milk P4 assay included a high, medium and low control with equal representation of each treatment in an assay. Three quality control samples were prepared from known concentrations of P4 (1 pg/mL) provided in ELISA kit for determination of extraction efficiency of P4 concentration in milk. The milk samples were randomly selected and 1 ml of each sample was extracted once with diethyl ether (1ml each time). The extraction was dried, re-suspended in 250 µL of assay buffer, vortexed twice and run directly in ELISA assay for P4 analysis. Once the concentration of P4 was confirmed to be sufficient in the milk samples, the remaining samples were then evaluated by non-extraction method directly in ELISA. All the milk samples had high, medium and low control. Each treatment was equally represented in each assay. Samples for a cow on each treatment were completed in single assay. The inter-assay coefficient of variation (CV) for P4 were 6.8%

(low), 8.3% (medium) and 2.7% (high) and the intra-assay CV for P4 were 7.6% (low), 5.4% (medium) and 4.9% (high).

Luteinising and follicle stimulating hormone assay

Plasma LH and FSH concentrations were measured by a double-antibody radioimmunoassay, as previously described for ovine gonadotropins (Salamonsen *et al*., 1973; Lee *et al*., 1976). Hormone concentrations and assay quality control data were calculated using the computer programme of (Burger *et al.,* 1972).

The LH assay used a primary antiserum raised in rabbit (NIH, AFP-240580) against bovine LH. Bovine LH (NIH, AFP-11118B) was used as the assay standard and for iodination.

Briefly, 100 ul assay buffer (0.5% BSA/0.03 M sodium phosphate monobasic/0.12 M sodium phosphate dibasic/0.1% sodium azide/0.1% triton-X), first antibody (1:1,400,00) diluted in 1:2000 normal rabbit serum (NRS) and 100 ul of iodinated bovine LH were added to duplicate plastic tubes containing either standard (0.5-50 ng/ml) or 300 ul bovine plasma. After incubation at 32°C for 24 h the antibody-bound hormone was separated from the free hormone by the addition of goat-anti-rabbit (GAR) (1:500). The tubes were incubated with second antibody overnight at 32°C before centrifugation (3,200 rpm; 30 min; 4°C), after which the supernatant was aspirated and the precipitate counted. All samples were assayed in a single assay. The sensitivity of the LH assay was 0.1 ng/ml and the intra-assay coefficient of variation was 7.3%.

The FSH assay used a rabbit anti-bovine FSH antiserum (NIH, AFP-7722291) and bovine FSH (NIH, AFP-9294C) was used as the assay standard and for iodination. Assay buffer (100 ul), first antibody (1:15,000) diluted in 1:2000 NRS and 100 ul of iodinated bovine FSH were added to duplicate plastic tubes containing either standard (2.5-320 ng/ml) or 300 ul bovine plasma. After incubation at 32°C for 24 h, second antibody (GAR 1:400) was added. The tubes were incubated at 32°C before the addition of 100 ul of 10% polyetheleneglycol then

incubated for 3 h at 4°C. This was followed by centrifugation (3,200 rpm; 30 min; 4°C), after which the supernatant was aspirated and the precipitate counted. All samples were assayed in a single assay. The sensitivity of the FSH assay was 0.1 ng/ml and the intra-assay coefficient of variation was 7.7%.

Statistical analysis

Initially, summary statistics by level and week of CDCO supplementation were computed to give means, standard deviations standard error, variance, minimum and maximum values that were scrutinised for any data entry errors (SAS, 2009). Testing for linear, cubic and quadratic orthogonal contrasts by regressing the dependent on explanatory variables was carried out using PROC REG. However, linear, quadratic and cubic orthogonal contrasts were tested for and found to be inconsequential. Therefore, repeated measures analysis of variance was employed fitting fixed effects and second-order interactions. Subsequently, P4, LH and FSH were analysed by repeated measures analysis of variance using PROC MIXED (SAS, 2009) utilising 1^{st}-order autoregressive covariance structure, and week of supplementation as the repeated effects. 1^{st}-order autoregressive covariance structure was utilised because it has homogeneous variances and correlations that decline exponentially with distance i.e. variability in measurement is constant regardless of when you measure it. The model included treatment, week of lactation and interaction between treatment and week of lactation as fixed effects, while base line hormone values and cows were fitted as covariate and random effects, respectively and the degrees of freedom were estimated by the Satterthwaite method (SAS, 2009). Variables of interest having significant treatment and or week of lactation effects are presented in Tables and Figures as LSM ±SEM and differences between means were considered significant at the $P < 0.05$ threshold unless otherwise stated. Significant differences and mean separations were carried out using Tukey's probability pairwise comparison tests.

Results

Table 6.2 Multi-trait analysis of variance (P-values) for fixed and interaction effects of treatment and week of supplementation on progesterone (P4), luteinising (LH) and follicle stimulating hormones (FSH) in Holstein-Friesian cows.

Hormones	Treatment Group				P-values		
	Control	Low	Medium	High	Treatment	Week	TRT*Week
FSH	0.172±0.02[a]	0.251±0.02[b]	0.367±0.07[c]	0.459±0.05[d]	**0.0002**	0.1972	0.9999
LH	0.391±0.20	0.426±0.21	0.349±18	0.459±05	0.4829	0.1364	0.4984
P4	1739.7±58.46	1867.3±62.91	1793.1±65.81	1797.5±58.26	0.0832	**0.0293**	0.4456

Row means within a variable bearing different superscripts significantly differ (P<0.05). All p-values in bold were significant (P<0.05). Follicle stimulating hormone (FSH, ng/mL), Luteinising hormone (LH, ng/mL), Progesterone hormone (P4, pg/mL) TRT, imposed treatment, Week, week of supplementation, Week*TRT, two way interaction of week of supplementation by imposed treatment. Each group had five cows.

Milk progesterone profile

The reported data focused on the observed temporal changes of mean milk P4 (picogram/ml; pg/ml) over the experimental period. It was observed that feeding CDCO to primiparous Holstein-Friesian had no significant effect (P>0.05) on P4 in milk (Table 6.2). However, as the week of supplementation progressed, CDCO diet supplement appeared to significantly affect (P<0.05) P4. The treatment by period interaction yielded no significant effect (P>0.05) on P4. Weekly trend for mean P4 concentration in milk was very similar across the groups (Figure 6.1). The observed temporal changes of milk P4 concentration from day -32 to day 32 indicated that regardless of the group, the secretion of P4 in milk was consistently similar throughout (Figure 6.2).

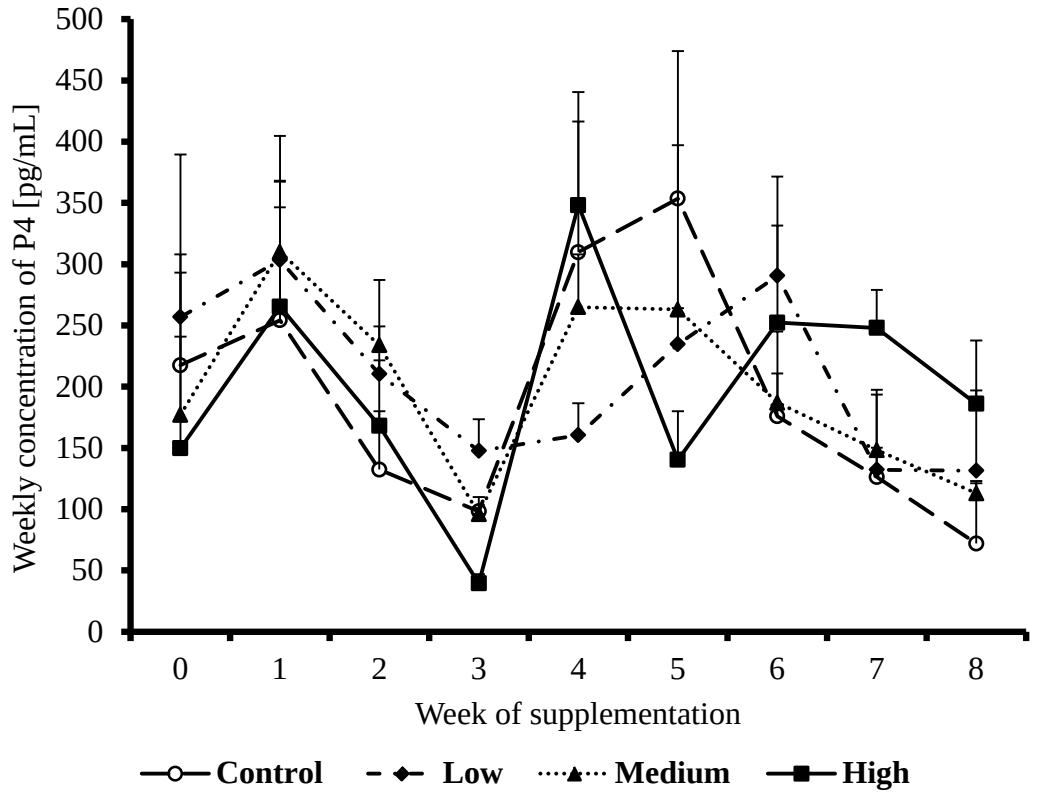

Figure 6.1 Weekly interaction between incremental level of CDCO supplement and week of supplementation on weekly progesterone (P4) concentration in milk of primiparous Holstein–Friesian cows grazing pasture for eight weeks; Pg, picogram (10^{12}).

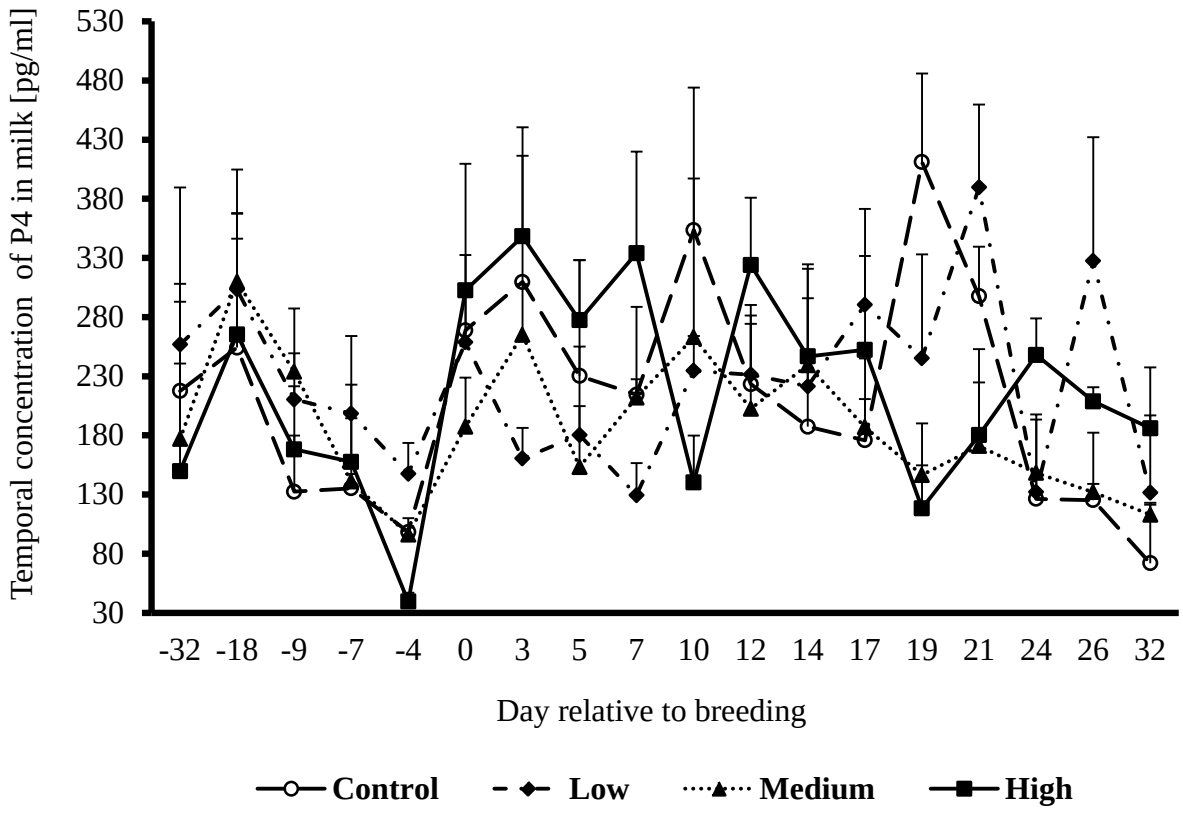

Figure 6.2 Temporal changes to progesterone profiles. Temporal changes in milk P4 concentrations during the treatment period for primiparous Holstein–Friesian. All values were least square means (±SEM). Note: -4 d, synchronization initiated; 0 d, heat detection began; 3 d, breeding initiated; Pg, picogram (10^{12}).

Plasma luteinizing hormone

There was no significant (P>0.05) effect of feeding CDCO to Holstein-Friesian cows on plasma LH (Table 6.2). In addition, week of supplementation had no significant effect (P>0.05) on plasma LH. The interaction between treatment and week was also not a significant (P>0.05) source of variation (Table 6.2). Weekly plasma LH trend was similar regardless of the group (Figure 6.3).

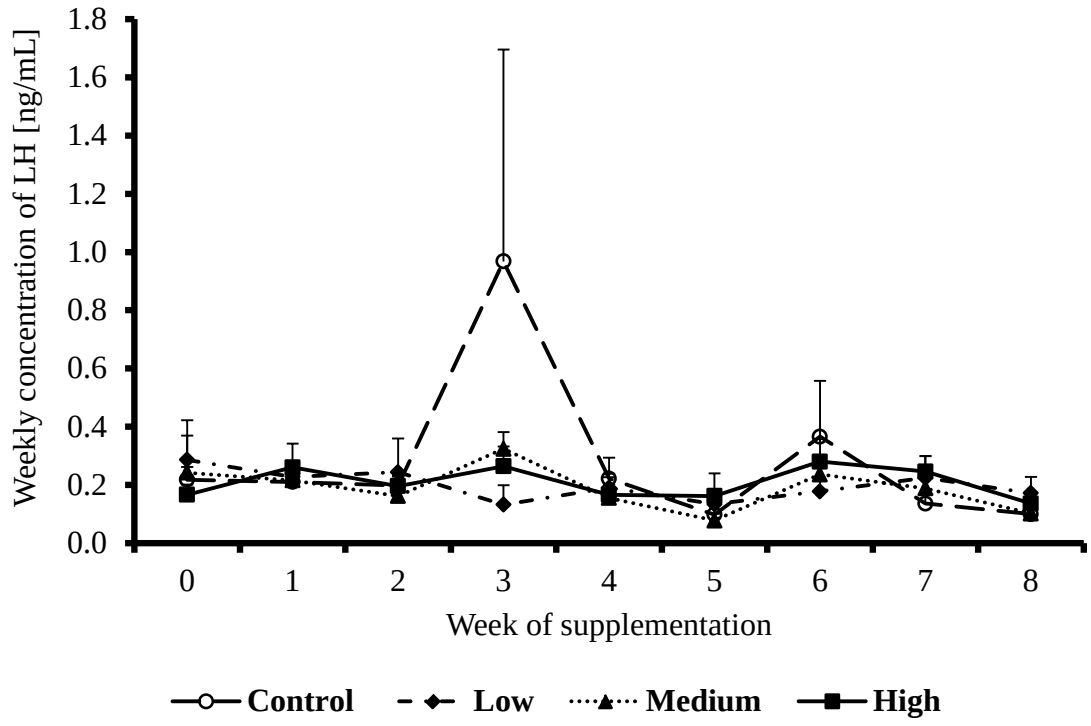

Figure 6.3 Weekly interaction between incremental level of CDCO supplement and week of supplementation on weekly plasma LH concentration of primiparous Holstein–Friesian cows grazing pasture for eight weeks.

Plasma follicle stimulating hormone

Result of the multivariate analysis of variance (P-values) for the effects of treatment and week of supplementation on plasma FSH is presented in Table 6.2. Treatment had significant ($P<0.05$) influence on plasma FSH, however, week of supplementation ($P>0.05$) and interaction between treatment and week of supplementation ($P>0.05$) did not significantly affect mean plasma FSH concentration. Cows in the high treatment group consistently produced greater plasma FSH throughout the weeks of supplementation. The trend was followed closely by the medium group cows; whereas the mean FSH concentrations of the low and control groups were lower (Figure 6.4). The cows in the high group (0.459±0.05 ng/mL) recorded the greatest total plasma concentration (eight weeks pooled values) of FSH, followed by the medium group (0.367±0.07), then the low group (0.251±0.02) in comparison to the control (0.172±0.02; Figure 6.5).

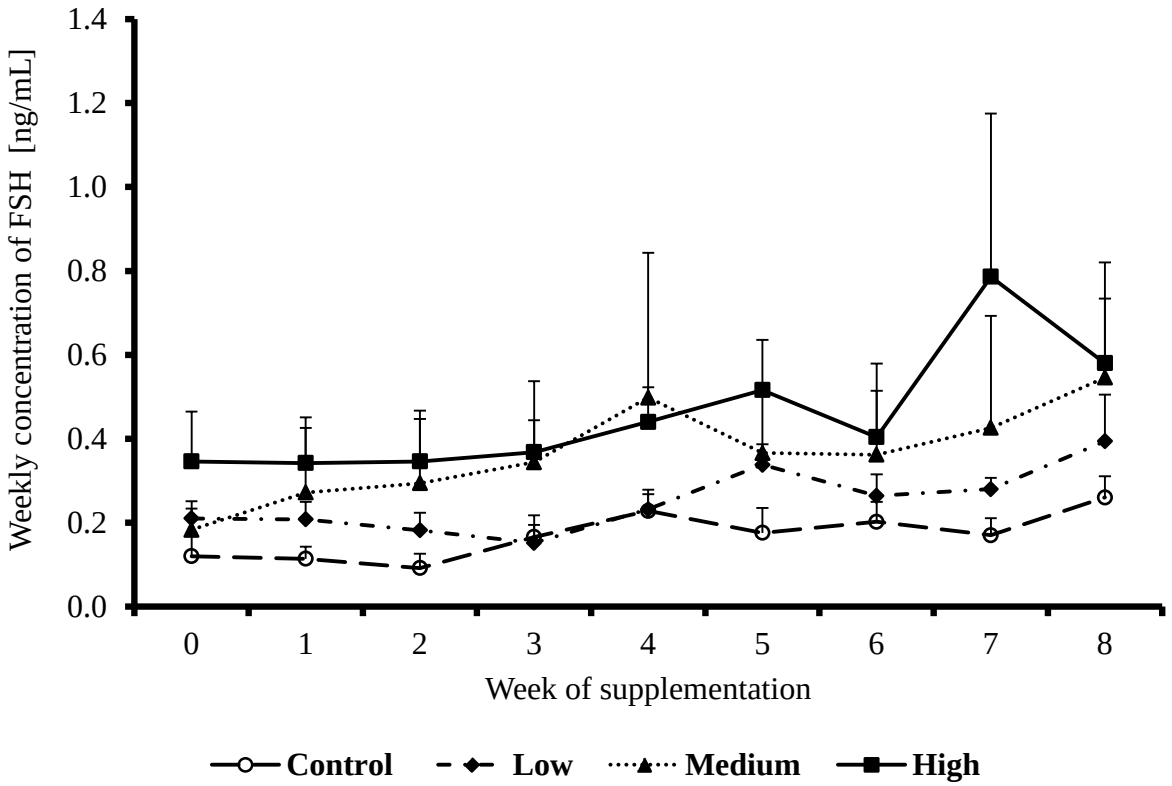

Figure 6.4 Weekly interaction between incremental level of CDCO supplement and week of supplementation on weekly plasma FSH concentration of primiparous Holstein–Friesian cows grazing pasture for eight weeks.

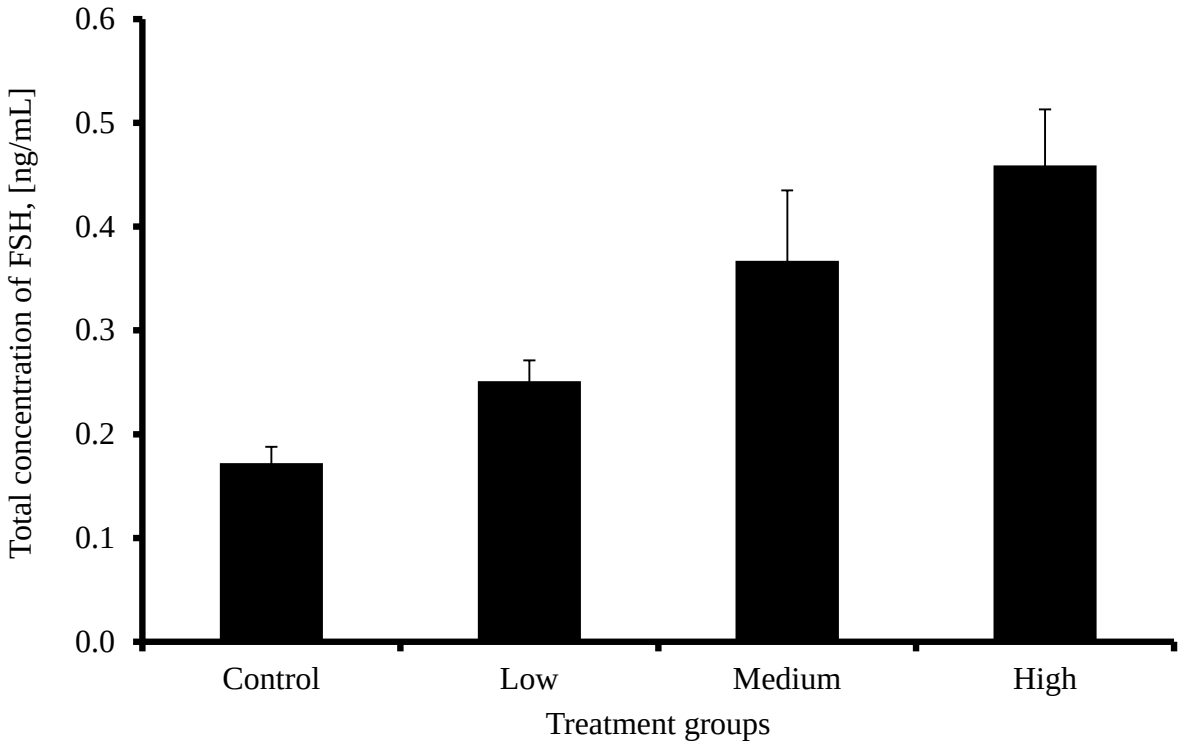

Figure 6.5 Total mean concentrations of FSH in plasma of primiparous Holstein–Friesian cows receiving 0 ml/kgDM (control), 25 ml/kgDM (low), 35 ml/kgDM (medium) and 50 ml/kgDM (high) levels of CDCO supplementation for eight weeks. A total mean concentration of FSH is the pooled values for the entire eight weeks.

Discussion

Milk progesterone profile

Feeding of diets containing canola oil did not influence the concentration of P4 in milk. Fat supplementations have been used efficiently to manipulate progesterone synthesis by altering the concentration of plasma cholesterol in dairy cows (Carroll et al., 1992). However, feeding dietary fat either increased (Coyral-Castel et al., 2010) or decreased (Robinson et al., 2002) progesterone synthesis, while in other studies no change was found (De Fries et al., 1998). Canola is known to contain phytosterol and triglycerol (Hamama *et al.,* 2003). Plants with high contents of phytosterol are comprised of low cholesterol (Hamama *et al.,* 2003). A previous study reported that phytosterol can significantly reduce cholesterol in humans with hypercholesterolemic conditions (Miettinen *et al*., 1995). Therefore, the lack of significant effect observed in the present study could be that CDCO was hypocholesterolemic (low cholesterol). In addition, no significant differences were observed between the unsupplemented and supplemented cows on the concentration of P4 in milk (Table 6.2). This indicates that the levels of fat used in the current study were inadequate, and that higher levels of CDCO than the current levels used in this study, are probably required. The current result also indicates that the effect of fat supplementation on P4 secretion might be dependent on the dosage of chemical composition (i.e. lipoproteins) and specific fatty acids of the experiment dietary fat. This argument is supported by Staples et al. (1998) who stated that the effect of fat on reproduction is independent of the cow's energy status, but rather depends on the specific fatty acid composition of the supplemented fat. Boken et al. (2005) also found that enhancement of plasma P4 concentration through fat supplementation was also accompanied by greater body weight loss, which further support the assertion that the effect of dietary fat supplement might be fatty acid specific.

Plasma luteinizing hormone and follicle stimulating hormone

The lack of changes in the LH concentration during the fat supplementation found in the present study is in agreement with that reported previously (Lucy *et al*., 1991). However, contrasting this study is that of Sklan et al. (1994) which showed that feeding primiparous cows with an inert fat source increases their plasma LH concentration during folliculogenesis. However, in the same study, they found that LH concentration was increased at the luteal stage in primiparous cows but not in multiparous cows. This seems to suggest that the effect of supplemented fats on LH synthesis may be elicited at different stages of oestrus cycle in cows at different parity and lactation stages. The non-significant effect of fat observed in the present study, however, could be due to the levels, form and duration of the supplementated fat. Boland et al. (2001) have also argued that a pulsatile LH secretion is not affected by short period dietary changes in ruminant. Most of the previous studies had a longer period of fat supplementation (Sklan *et al*., 1994). In the present study, feeding only took place for eight weeks. It seems that longer feeding (>8 weeks) might be required for grazing primiparous Holstein Friesian cows. The levels of CDCO used in the current study did not exceed 50 ml kg^{-1}DM. This is based on calculations, levels above 50 ml kg^{-1}DM would have exceeded the 7% critical level of total fat allowed in the diet of grazing cows. However, greater levels of CDCO supplementation than utilized in this study might be required. The energy level of supplemented fat is usually essential for gonadotropins synthesis (Randel 1990). The fat source, CDCO, used in this study had similar metabolisable energy to the control feed (4.08 vs 4.07 MJ/kg DM; Table 6.1). Randel (1990) argued that a diet low in energy leads to low pulsatile release of LH. Therefore, greater level of CDCO with greater metabolisable energy than currently applied needs to be considered. However, in the present study the concentration of plasma LH was determined from weekly samples. Plasma LH is secreted in a pulsatile manner and the current plasma LH concentration result from weekly

samples may not be that informative, because samples may have been collected from between or during a pulse. However, the lack of significant effect of treatment on LH suggests that fat supplements may have conflicting effects on gonadotropin hormones, where having a significant effect on one might not be true for the other.

Feeding CDCO to primiparous Holstein-Friesian increased the mean plasma concentration of FSH in the present study. A previous study that fed three levels of total dietary fat to dairy cows found no significant differences in plasma FSH regardless of the diet (Beam & Butler, 1997). Availability of FSH in the ovary is associated with the growth and development of ovarian follicles through a cycle of positive and negative feedback mechanisms between the anterior pituitary, hypothalamus and ovary axis (Squires, 2010). In another study, feeding soybean to beef cows increased the number of medium size follicles compared to fish oil and saturated fat treatments (Thomas *et al.*, 1997). Vegetable oil contains more LA (an ω-6 FA) as compared with eicosapentaenoic and docosahexaenoic acids (ω-3 fatty acids) in fish oil, which suggests that specific LA within the supplemented fat do have specific physiological functions on the secretion of FSH (Gulliver *et al*., 2012). It is suggested that LA assists the synthesis of PGF2α (Gulliver *et al*., 2012). Increased concentration of PGF2α at uterine level causes the regression of formed corpus luteum, thus stimulating the return to oestrus cycle (Gulliver *et al*., 2012). As a consequence, the secretion of FSH is initiated in the anterior pituitary gland to facilitate recruitment of ovary follicles (Lucy *et al*., 1991). On the other hand, glucose is also spared at the mammary gland as a result of milk fat depression following fat supplementation (Bauman & Griinari, 2003). Consequently, excess glucose arising from the mammary gland is suggested to be channelled to the hypothalamus-pituitary axis to boost the energy levels and to enable secretion of more gonadotropin hormones (Staples *et al.*, 1998). It has been suggested that fat supplementation might have a direct

effect on the hypothalamo-pituitary-ovary axis, which would affect the availability of gonadotropins (Lucy *et al*., 1991).

Conclusion

Fat supplementation has been reported to improve reproductive performance in dairy cattle by altering the concentrations of key reproductive hormones. In the current study, feeding CDCO to primiparous Holstein Friesian cows for eight weeks increased the plasma FSH as the supplement levels increased. However, no changes were observed in the concentrations of P4 and LH between treatments, which suggests that greater levels of CDCO than were currently used might be required to alter the profiles of P4 and LH in cows. However, the present study does provide evidence that CDCO supplementation to primiparous Holstein-Friesian cows at 25 ml/kgDM, 35 ml/kgDM and 50 ml/kgDM in a pasture-based system could enhance the circulating plasma FSH without affecting the concentrations of LH and P4 in plasma and milk under Australian pasture-based conditions. This could have practical beneficial implications for reproductive success in pasture-based dairy systems, considering that FSH is essential for growth, development and maturation of ovarian follicles. However, due to non-significant differences between supplemented and unsupplemented cows in P4 and LH, we propose that higher levels of CDCO than the current levels used in this study, are probably required. Furthermore, poor reproductive performance experienced by primiparous Holstein-Friesian cows grazing pasture might not be due to atypical hormonal profiles, because other factors may be involved. The full extent of how lipid supplementation alters the dynamics of steroids and gonadotropic hormones in dairy cows still eludes us and warrants further investigation into other molecular genetic factors such as gene expression and mRNA profiles of supplemented cows to provide a better understanding of CDCO's role in future applications as a dietary fat supplement for lactating cows.

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Chapter 7 : Effect of supplementation with crude degummed canola oil on the expression of fat-related genes involved in reproduction and lipogenesis in primiparous Holstein-Friesian dairy cows in a pasture-based system

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Abstract

The effect of oil-rich supplements on the expression of genes involved in reproduction and lipogenesis in pasture-based dairy cows is currently unknown, or at best, scanty and limited to impacts on cow liveweight, body condition score, milk composition, fatty acid and plasma metabolite profiles only. This research investigated the effect of dietary inclusion of incremental levels of CDCO on the expression of Arylalkylamine N-acetyltransferase (AANAT), B-cell translocation gene-2 (BTG2) and Fatty Acid Synthase (FASN) genes involved in reproduction and lipid synthesis. We tested the hypothesis that post-partum supplementation of primiparous Holstein-Friesian cows with dietary CDCO in a pasture-based system will alter the relative mRNA abundance and expression of AANAT, BTG2 and FASN genes associated with lipid metabolism. A random allocation of twenty primiparous Holstein-Friesian dairy cows into four treatment groups comprising wheat-based pelleted basal diet with no supplemental CDCO (control), or a wheat-based pelleted basal diet with CDCO added at 25 ml/kgDM (low), 35 ml/kgDM (medium) and 50 ml/kgDM (high) was utilized in a ten-week experimental feeding trial including two weeks of adjustment. Blood samples were subjected to mRNA extraction and reverse transcription using quantitative polymerase chain reaction (RT-qPCR) to assess the mRNA expression levels of the AANAT, BTG2 and FASN genes. Both level and duration of supplementation with CDCO were significant sources of variation ($P<0.05$) that influenced BTG2 expression, while the expressions of AANAT and FASN genes were unaffected ($P>0.05$). Cows in the high (0.67 fold), medium (0.87 fold) and low (0.56 fold) levels of oil treatments had lower expressions of BTG2 gene compared to the control (1.0 fold) group of cows. The supplementation of cows with lipid-rich feeds could be utilised as a dietary manipulation tool to down-regulate the expression of BTG2 gene and its anti-proliferative attributes. The low expression of BTG2 might be important when the reproductive system of cows is recovering from the

effect of gestation and new cell growth is required. The suppression of FASN gene expression can be beneficial in sparing energy from milk fat synthesis and re-directing the surplus to non-mammary tissues *in vivo*. However, severe milk fat depression may be economically undesirable to Tasmanian dairy farmers because of its contribution to total milk solids upon which milk prices are based. These findings highlight the important role of supplementary nutrition in altering reproductive and lipogenic gene expression in lactating primiparous cows.

Keywords: crude degummed canola oil; Aralkylamine N-acetyltransferase; B-cell translocation gene 2; Fatty acid synthase; Primiparous Holstein-Friesian cows

Introduction

Nutritional attempts to remedy infertility are of interest to the dairy industry (Chagas *et al.,* 2007) because the antagonistic relationship between high milk production and fertility in modern, high genetic-merit cows has led to a gradual but progressive, decline in reproductive performance in diverse dairy production systems around the world. Prolonged calving intervals along with embryonic losses and postpartum anovulatory intervals are some of the major causes of infertility in cows (Rocha *et al.,* 2010). In a typical pasture-based dairy system, different sources of lipids fed to lactating cows have been studied to primarily increase the energy density of the diet in order to enhance milk production when negative energy balance peaks (Hutchinson *et al.,* 2011). Research findings suggest that dietary supplementation with fat sources containing adequate proportions of unsaturated fats could potentially improve fertility in high merit dairy cows (Santos *et al*., 2008). Therefore, a new, effective and long-term nutritional strategy that can assist in a better understanding of nutrition-fertility interactions in pasture-based systems is a potential solution to the subfertility problem in dairy cows.

Lipids also epitomize an effective nutritional approach for modifying milk fat composition (Staples *et al.,* 1998) to favour an elevated profile of beneficial polyunsaturated fatty acids (Otto *et al.,* 2014). Lipids also play a crucial role in regulating the expression of genes essential for fertility and *de novo* fat synthesis in dairy cows. It has been shown that dietary fats containing trans-10, cis-12 conjugated linoleic acid (CLA) cause milk fat depression by inhibiting the expression of FASN gene (Hutchinson *et al.,* 2011) which is known to play a central role in the biosynthesis of fat in the mammary gland of mammals (Roy *et al.,* 2006). Although FASN is an important gene involved in lipogenesis, there is only limited published information about its expression in fat-supplemented cows in a pasture-based dairy

production system. Where such studies were conducted, results have been conflicting and inconsistent (Vahmani *et al.,* 2014) and warrant further research.

Arylalkylamine N-acetyltransferase (AANAT) is an essential gene for melatonin biosynthesis (Öner *et al.,* 2014). Melatonin is directly associated with optimal functioning of the ovary, where it regulates the hypothalamic-pituitary-gonadal axis to instigate folliculogenesis and steroidogenesis (Chowdhury *et al.,* 2013; Fiske *et al.,* 1984). However, previous studies on AANAT have focused mainly on humans, in spite of AANAT being an important gene controlling reproduction in other mammals (Soria *et al*., 2010). Currently, there is limited information on the expression of AANAT gene in dairy cows, especially with regards to fat supplementation, thus creating an important knowledge gap that this study intends to fill.

B-cell translocation gene-2 (BTG2) is an anti-proliferative gene that regulates cell cycle growth and BTG2 research investigations have been limited to cancer studies (Choi *et al*., 2013). A previous study has reported that the antiproliferative trait of BTG2 gene is essential during mammal ovulation, which demonstrates the essentiality of BTG2 gene in mammal's ruminant reproduction (ovulation; Park *et al*., 2013). Published information on the expression and function of the BTG2 gene in dairy cattle, especially when supplemented with dietary lipids in a pasture-based system, are to our knowledge, either non-existent or at best, scanty. The above mentioned genes are related with differences in total fatty acid content in animal tissues and the protection of long chain polyunsaturated fatty acids through the prevention of peroxidation (Perez *et al.*, 2010).

Understanding the mechanism underpinning the impact of dietary fat intake on the reproductive sequences from oestrous to conception in cows could revolutionise how nutrition is managed in dairy farms to improve reproductive performance. It will also assist researchers in unravelling the mystery behind the current global and gradual, but progressive, decline in dairy cow fertility. In this regard, further studies are required to elucidate the

intricate biological mechanisms involved with feeding dietary fats to grazing cows and their effects on lactation and fertility traits. This will enable dairy farmers make informed choices and tailored decisions when feeding lactating cows with specific dietary fat supplements. We hypothesized in this study that supplementation with CDCO would affect the expression of genes involved in reproductive functions (AANAT, BTG2) and *de novo* fatty acid synthesis (FASN) in primiparous Holstein-Frisian cows grazing under similar environmental conditions. Therefore, the primary objective of this study was to determine the relative abundance and expression of genes encoding proteins required for optimal reproduction and *de novo* lipogenesis in pasture-based lactating cows subjected to zero, low, medium and high levels of dietary supplementation with CDCO.

Materials and Methods

The use of animals and procedures performed in this study were all approved by the University of Tasmania Animal Ethics Committee (Permit No AA0012583), and were conducted in accordance with the 1993 Tasmanian Animal Welfare Act and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Site and climatic conditions

The experiment was carried out at the University of Tasmania's Dairy Research Centre, Tasmanian Institute of Agriculture (TIA) Elliot Dairy Research Farm in Somerset, North-Western Tasmania, Australia, from September to November 2012. Tasmania is Australia's smallest state with a land size of 68,000 square kilometers and located within the cool, temperate, climatic zone at latitude 42˚ South and longitude 147˚ East. It is characterized by four distinct seasons - winter, autumn, spring and summer. The experiment was carried out in spring when the annual rainfall was 2500 mm and humidity was approximately 60%.

Animals and treatments

The physical condition and energy status of the experimental cows were visually assessed based on BCS on a scale of 1-8 (DPI, 2003; Stockdale, 2001). Twenty primiparous, spring-calving, purebred, Holstein-Friesian cows (average liveweight of 400 ± 40 kg, BCS 4 ± 1 and 40 ± 8 DIM, were randomly allocated into 1 of 4 treatment groups of supplementation with CDCO classified as low (25 ml/kgDM), medium (35 ml/kgDM), high (50 ml/kgDM) and the control (no CDCO-0 ml/kgDM). For the supplementation trial, a complete randomise experimental design (CRD) was applied. This replicated herd of cows (n=5 per treatment group) receiving CDCO supplements was placed under the same management and rotated in electric fenced paddocks with the Control cows offered wheat-based pellets without CDCO. Together, the animals had access to 3000 kgDMha^{-1} of forages, a mixture of ryegrass (*Lolium perenne*), cocksfoot (*Dactylis glomerata*), and white clover (*Trifolium repens)* pasture grazed at the two-leaf stage. Water was offered *ad libitum.* The current level of CDCO was calculated based on 7% total fat recommended in the diet of grazing cows (Schroeder *et al*., 2004). Each cow received 6 kg of the pelleted supplements daily for eight weeks, after two weeks of adjustment. Supplements were offered to cows in two splits; morning (3kg) and evening (3 kg) milking sessions at 05:00 h and 15:00 h. There were no orts from any of the groups. The exact pasture intake was difficult to estimate as the case is under grazing conditions.

Feed chemical composition and analysis

Dry matter (DM) content of the basal and experimental diets was determined by drying samples to a constant temperature at 65°C in a fan forced oven, finely ground to pass through a 2mm sieve using Laboratory Mill (Thomas Model 4 Wiley® Mill; Thomas Scientific), and further drying at 105^{0}C for 24 h. The DM was computed as the difference between the initial and final weights of samples. Ash content was determined by combusting samples in a

furnace at 600°C for 8 hours. NDF and ADF contents were measured using an Ankom Fiber Analyzer (ANKOM220; ANKOM Technology, USA). Nitrogen content was determined using a Thermo Finnigan EA 1112 Series Flash Elemental Analyzer and the values multiplied by 6.25 to give the CP percentage. Ether extract (EE) was determined using an Ankom fat/oil extractor (ANKOM^{XT15}; ANKOM Technology, USA). Metabolisable energy was calculated as per Van Es (1975). The chemical compositions of the treatment, control and basal feeds are presented in Table 7.1.

Table 7.1 Chemical composition of the experimental, control and basal feeds.

Chemical composition (%DM)	Feeds		
	Control (No canola oil)	Treatment (Canola oil)	Basal diet (Pasture)
MC	9.1	8.2	5.5
DM	90.9	91.8	94.5
ADF	9.0	8.0	27.7
NDF	21.1	20.0	45.9
EE	2.1	6.2	3.0
Ash	8.9	9.7	9.3
NFC	59.0	52.8	23.9
OM	91.1	90.3	90.7
CP	10.4	12.7	21.0
ME (MJ/kg DM)	4.07	4.08	3.99

All feeds were analysed based on a dry weight basis; Moisture content (MC), Dry matter (DM), organic matter (OM), neutral detergent fibre (NDF), acid detergent fibre (ADF), non-fibrous carbohydrate (NFC), ether extract (EE), crude protein (CP) and Metabolisable energy (ME). Treatment = feed with added canola oil. Control = feed without canola oil, Basal diet = mainly mixed ryegrass pasture.

Basal and supplement fatty acid analysis

The fatty acid profiles of both basal and supplementary feeds were analysed by gas liquid chromatography (GLC) and presented in Table 7.2. The detailed procedure had been previously described and published (Otto *et al.*, 2014).

Table 7.2 Fatty acid concentration as a percentage of total fatty acids of treatment, control and basal feeds.

Fatty acid (%)	Feed components		
	Control (No canola oil) %	Treatment (canola oil) %	Basal (Pasture) %
12:0	0.00	0.00	0.05
14:0	0.10	0.09	0.10
15:0	0.20	0.13	0.20
16:1	0.00	0.00	1.00
16:0	32.10	26.10	10.00
17:0	0.20	0.18	0.10
18:3ω 6	0.00	0.03	0.00
18:4ω 3	0.00	0.00	0.90
18:2ω 6 LA	17.70	6.86	9.10
18:3ω 3 ALA	1.60	0.48	64.30
18:1ω 9c	16.50	41.90	4.40
18:1ω 7t	0.20	0.10	0.20
18:0	3.80	3.83	2.20
18:2CLA	0.10	1.48	0.00
19:0	0.90	3.47	0.10
20:4ω 6 ARA	0.00	0.01	0.00
20:3ω 6	0.40	1.82	0.80
20:4ω 3 ETA	0.40	0.22	0.10
20:2ω 6	1.40	1.45	0.00
20:0	0.80	1.38	0.40
22:5ω 6	0.30	0.04	0.10
22:6ω 3 DHA	0.20	0.03	0.00
22:4ω 6	0.20	0.00	0.00
22:5ω 3 DPA	0.90	0.00	0.00
22:0	1.80	1.86	1.50
24:0	1.10	1.30	0.90
*t*SFA	41.20	38.64	16.45
*t*MUFA	23.30	48.74	8.00
*t*PUFA	35.00	12.62	75.40
ω-3 PUFA	14.90	0.93	65.40
ω-6 PUFA	20.10	10.24	10.10
ω-3 LC-PUFA	13.30	0.45	0.20
Other FA	11.80	0.20	0.10

∑*t*SFA is the sum of 12:0, 13:0, i14:0,14:0, i15:0, a15:0,15:0, i16:0, 16:0, i17:0, 17:0, i18:0, 18:0, 19:0, 20:0, 20:0, 22:0, 24:0; ∑*t*MUFA is the sum of 14:1ω -5c, 15:1ω-6c, 16:1ω -9c, 16:1ω -7c, 16:1ω -7t, 16:1ω -5c, 16:1,17:1ω -8+a17:0, 17:1ω-6c, 18:1ω -9c, 18:1ω -7c, 18:1ω -7t, 18:1ω -5c, 18:1a, 18:1b , 20:1ω -11c, 20:1ω -9c, 20:1ω -7c, 20:1ω -5c, 22:1ω -11c, 22:1ω -9c, 22:1ω -7c, 24:1ω -11c, 24:1ω -9c, 24:1ω -7c; ∑*t*PUFA is the sum of 18:3ω-6, 18:4ω-3, 18:2ω-6, 18:3ω-3, 18:2CLA, 20:4ω-6, 20:5ω-3, 20:3ω-6, 20:4ω-3, 20:2ω-6, 22:5ω-6, 22:6ω-3, 22:4ω-6, 22:5ω-3; ∑ω-3 LC-PUFA is the sum of 20:5ω-3, 20:4ω-3, 22:6ω-3, 22:5ω-3; ∑ω-3 PUFA is the sum of 18:4ω-3, 18:3ω-3, 20:4ω-3, 20:5ω-3, 22:6ω-3, 22:5ω-3; ∑ω-6 is the sum of 15:1ω-6, 17:1ω-6, 18:2ω-6, 18:3ω-6, 20:4ω-6, 20:3ω-6, 20:2ω-6, 22:5ω-6, 22:4ω-6. *t*SFA= total saturated fatty acids, *t*MUFA= total monounsaturated fatty acids, *t*PUFA= total polyunsaturated fatty acids, ω-3 FA= total omega-3 fatty acids, ω-6 FA=total omega-6 fatty acids; ω-3 LC-FA=total omega-3 long chain fatty acids, Other FA= is the sum of unknown FA; Control= feed with no added canola oil; Treatment= feed with canola added; basal= mixed ryegrass pasture.

Blood sample collection

Blood samples were collected from all experimental cows by coccygeal venipuncture into vacutainers containing heparin after the morning milking (05:00 h) on the day before the initiation of supplementation with CDCO and in week eight at the conclusion of the experiment. More frequent blood sample collection interval was restricted by the terms and conditions of the Animal Ethics Permit No AA0012583 granted by the University of Tasmania Animal Ethics Committee. The samples were immediately frozen in -20ºC and transported to the laboratory for further storage at -80ºC until RNA extraction.

Ribonucleic Acid extraction and cDNA synthesis

Frozen blood samples were thawed and utilised for the isolation of total RNA using TRIzol® Plus RNA Purification Kit (Life Technologies Pty Ltd. Victoria, Australia). A tissue lyser (Qiagen Ltd., Crawley, UK) was used to homogenise the sample in TRIzol® Reagent. Total RNA quantity and quality was measured using the NanoDrop 8000 spectrophotometer (NanoDrop, Wilmington, DE, USA). RNA that had an absorbance (A260/280) reading between 1.8 and 2 was deemed of good quality. The RNA samples were treated with PureLinkTMDNase (Life Technologies Pty Ltd. Victoria, Australia) and purified using the RNeasy1 Mini Kit (Qiagen Ltd, NSW, Australia). DNase-treated and purified total RNA was then reverse transcribed to cDNA with Mixed Oligo dT/Random Hexamer Primers using the Tetro cDNA Synthesis Kit (Bioline Pty Ltd. NSW, Australia) according to the manufacturer's instructions.

Primer design and reference gene selection

All candidate and reference gene primers (Table 7.3) were designed using the Primer3 web-based software program (http://frodo.wi.mit.edu/primer3/) from GeneWorks Pty Ltd, SA, Australia). Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) was used to check for the

specificity of the primers. The validity of all primers was confirmed using a serial dilution of pooled cDNA to create a standard curve. Subsequently, the amplified PCR products were sequenced to confirm their primer specific identity (Beckman Coulter CEQTM 8000 Series Genetic Analysis System). The mRNA abundance was determined using highly stable reference genes. The normalisation of expression data for the target genes Aralkylamine N-acetyltransferase (AANAT), B-cell translocation gene 2 (BTG2), and Fatty acid synthase (FASN) utilised two reference genes, Ubiquitin C (UBC) and Peptidyl-prolyl cis-trans isomerase-A (PPIA). A good selection criterion of reference genes was an expression ratio that was constant across all samples. The software program geNorm, version 3.5 (Vandesompele *et al.*, 2002), was used to calculate, confirm and validate the expression stability (M-value) of the reference genes.

Table 7.3 Real-time quantitative PCR (qPCR) primers.

Gene symbol	qPCR Primers		T_a	Amplicon Size (bp)
	Forward Primer	Reverse Primer		
AANAT	ACTGACCTTCACGGAGATGC	TTCACTCATTCTCCCCGTTC	60	211
BTG2	CTGGAGGAGAACTGGCTGTC	AAAACAATGCCCAAGGTCTG	60	194
FASN	GTGTGGTACAGCCCCTCAAG	ACGCACCTGAATGACCACTT	60	110
UBC	CGTCTTAGGGGTGGCTGTTA	AAATTGGGGTAAATGGCTAGA	60	90
PPIA	TCATTTGCACTGCCAAGACTG	TCATGCCCTCTTTCACTTTGC	60	72

Aralkylamine N-acetyltransferase=AANAT, B-cell translocation gene 2=(BTG2, Fatty acid synthase=FASN, Ubiquitin C=UBC, Peptidyl-prolyl cis-trans isomerasa=PPIA, Ta=Empirical annealing Temperature.

Quantitative real time PCR (qPCR)

Following reverse transcription, cDNA quantity was determined and standardised to the required concentration for qPCR. Triplicate 20 µL reactions were carried out in a 72-well Rotor-Gene (QIAGEN GmbH, Hilden, Germany), containing 4 µL cDNA (50 ng), 10 µL 2× SensiFAST SYBR No-ROX Mix (Bioline Pty Ltd., NSW, Australia), 4.4 µL DEPC H2O, and 0.8 µL forward and reverse primers (100 fmol). Assays were performed using the Rotor-

Gene 3000 (QIAGEN Pty Ltd., VIC, Australia) with the following cycling parameters: 95°C for 2 min polymerase activation; 40 cycles of 95°C for 5 s denaturation, 60°C for 10 s annealing and 72°C for 5 s extension. Gene expression levels were recorded as Ct values (i.e., the number of PCR cycles at which the fluorescence signal was detected above the threshold value) and all samples were run in triplicates. Amplification efficiencies were determined for all candidate and reference genes using the formula E = 10^(−1/slope), with the slope of the linear curve of cycle threshold (Ct) values plotted against the log dilution as per (Higuchi *et al.,* 1993). Primer concentrations were optimised for each gene and disassociation curves were examined for the presence of a single PCR product. The efficiency of the reaction was calculated using a 5-fold serial dilution of cDNA and generation of a standard curve. All PCR efficiency coefficients were between 1.7 and 1.8 and therefore deemed acceptable. The Rotor-Gene 3000 (version 6.0.16) (QIAGEN Pty Ltd., VIC, Australia) was used for efficiency correction of the raw Ct values. This process involved an inter-plate calibration based on a calibrator sample included on all plates, averaging of replicates, normalisation to the reference gene and the calculation of quantities relative to the highest Ct and $\log^2$ transformation of the expression values for all genes. A PCR efficiency coefficient between 1.7 and 1.8 was considered adequate. The mathematic model used to determine the expression level of the target gene in comparison to the reference gene is given below as per Pfaffl (2001).

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(control - sample)}}{(E_{\text{ref}})^{\Delta \text{CP}_{\text{ref}}(control - sample)}}$$

Statistical analysis

Initially, summary statistics by level and week (duration) of CDCO supplementation were computed to give means, standard deviations, standard error, variance, minimum and maximum values that were scrutinised for any data entry errors. Testing for linear, cubic and quadratic orthogonal contrasts by regressing the dependent on explanatory variables was carried out using PROC REG (SAS, 2009). However, the linear, quadratic and cubic orthogonal contrasts were all found to be inconsequential. Therefore, repeated measures analysis of variance using PROC MIXED (SAS, 2009) was employed fitting fixed effects of treatment, week of supplementation and their second-order interactions on the expressions of AANAT, BTG2 and FASN genes. The 1^{st}-order autoregressive covariance structure was utilised, and the level of supplementation were fitted as the repeated effects and cows as random effects. 1^{st}-order autoregressive covariance structure was utilised because it has homogeneous variances and correlations that decline exponentially with distance i.e. variability in measurement is constant regardless of when you measure it. The degrees of freedom were estimated by the Satterthwaite method (SAS, 2009). Variable means are presented in Figures as LSM ±SEM. Tukey's pairwise comparison test was utilized in establishing differences between means using the $P < 0.05$ threshold for significance unless otherwise stated.

Results

Table 7.4 and Figures 7.4, 7.5 and 7.6 represent the relative mRNA abundance and expressions of *AANAT*, *BTG2* and *FASN* genes, and these are individually highlighted as follows.

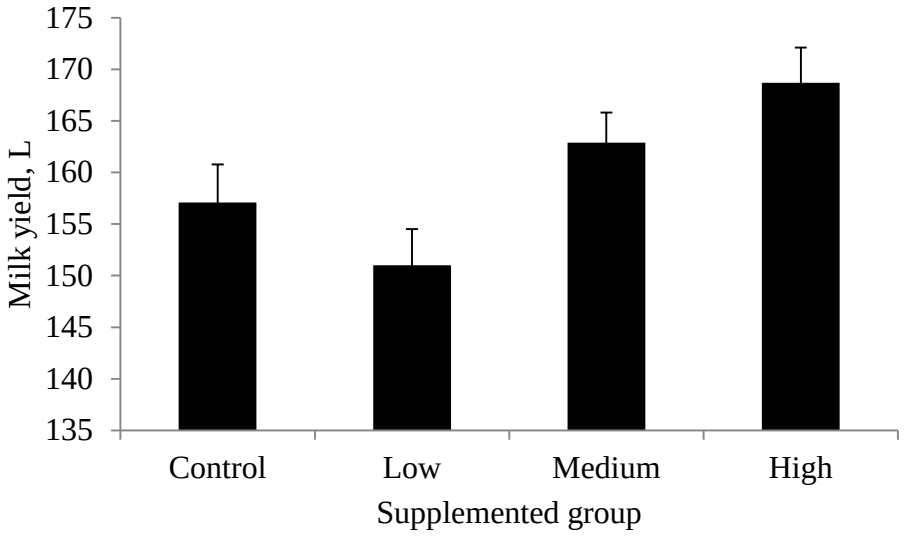

Figure 7.1 Effect of supplementing primiparous Holstein-Friesian dairy cows with CDCO on milk yield. Data presented is for eight weeks of collection, starting from week 0 to 8. Values are means ± SEM.

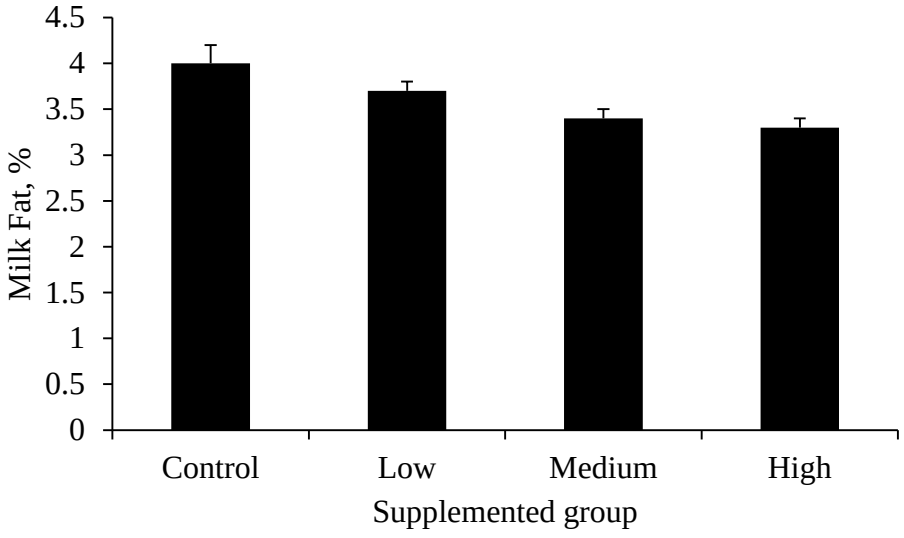

Figure 7.2 Influence of supplementing Holstein-Friesian dairy cows with CDCO on milk fat percentage. Statistical analysis was performed using data from week 0 to 8. Values are means ± SEM

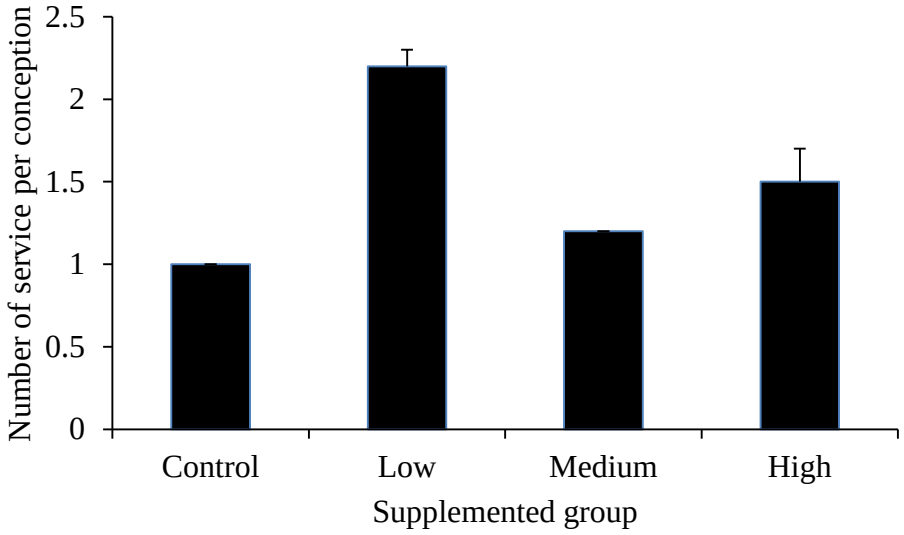

Figure 7.3 Number of services per conception in primiparous Holstein-Friesian dairy cows supplemented with CDCO for eight weeks. Values are means ± SEM

The effects of supplementation with CDCO on milk yield, milk fat and number of services per conception are presented in Figures 7.1, 7.2 and 7.3, respectively. Cows receiving 50 mL kg^{-1}DM (High) of canola oil produced more milk (168.7±3.4 vs 157.1±3.7 Litres) with a lower fat percentage (3.3±0.1 vs 4.0±0.2 %) than unsupplemented cows in the control treatment group (0 mL kg^{-1}DM) as depicted in Figures 7.1 and 7.2.

Aralkylamine N-acetyltransferase (AANAT)

Dietary supplementation of primiparous Holstein-Friesian cows with CDCO had no effect ($P>0.05$) on the expression of *AANAT* gene across the groups (Figure 7.4). As the week (duration) of supplementation progressed, the impact of CDCO supplements and interaction with duration of supplementation was insignificant on the expression of *AANAT* gene (Table 7.4).

Table 7.4 Multi-trait analysis of variance (p-values) for fixed and interaction effects of treatment and week of supplementation on the relative mRNA expression of Arylalkylamine-N-acetyltransferase (AANAT), B-cell translocation gene-2 (BTG2), Fatty acid synthase (FASN) genes in grazing Holstein-Friesian dairy cows.

Effect	Genes		
	AANAT	BTG2	FASN
TRT	0.2019	**0.0495**	0.9289
Week	0.2713	0.1818	0.3314
Week*TRT	0.4956	**0.0107**	0.6647

All p-values in bold were significant (P<0.05). Arylalkylamine-N-acetyltransferase (AANAT), B-cell translocation gene-2 (BTG2), Fatty acid synthase (FASN), TRT, treatment, Week, week of lactation, Week*TRT, interaction between week of supplementation and treatment.

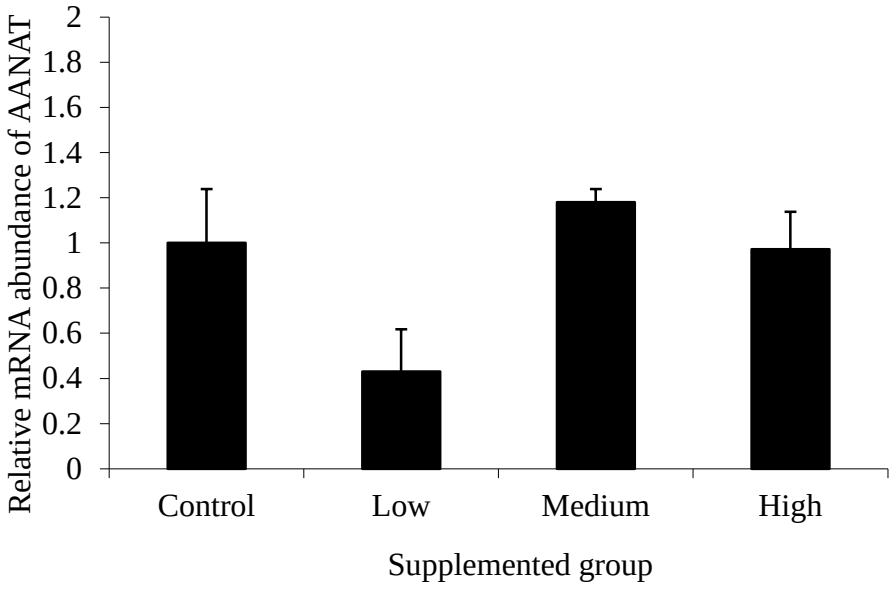

Figure 7.4 Effect of dietary supplementation of grazing cows with CDCO on mRNA expression of AANAT gene. Values are expressed as the geometric mean of the housekeeping genes/average Cp values for each gene. Values are means ± SEM

B-cell translocation gene 2 (BTG2)

It was evident that both treatment and treatment by week interactions were significant sources of variation that influenced the expression of *BTG2* gene (P<0.05; Table 7.4). However, week of supplementation alone had no influence (P>0.05) on *BTG2* gene expression. Cows receiving dietary supplementation with CDCO experienced suppression of *BTG2* gene expression (Figure 7.5). The cows in the control group recorded the greatest mRNA abundance of *BTG2* (1.00 fold), followed by the medium group (0.87 fold), then the high group (0.67 fold) and finally the low group (0.56 fold).

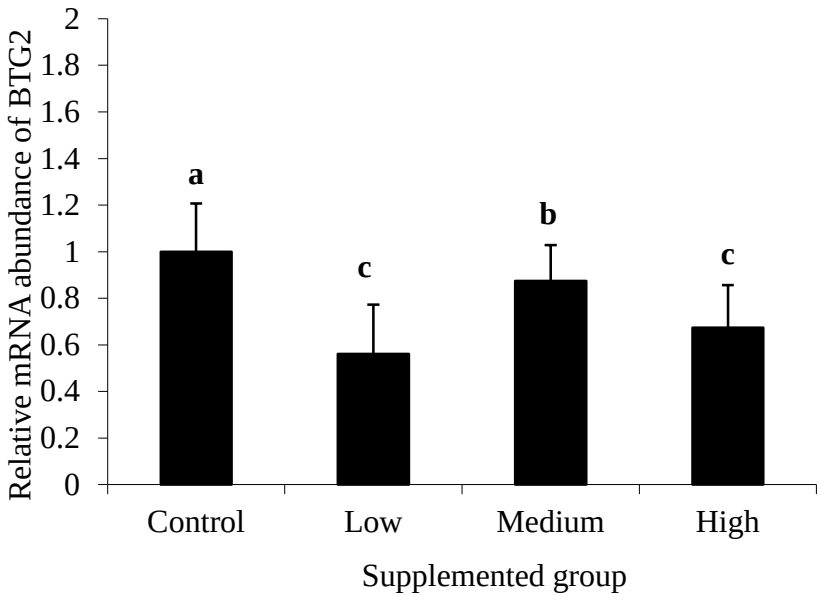

Figure 7.5 Effect of supplementation with CDCO on mRNA expression of the BTG2 gene in grazing cows. Values are expressed as the geometric mean of the housekeeping genes/average Cp values for each gene. Values are means ± SEM

Fatty acid synthase (FASN)

Differences in CDCO content in the supplemented primiparous Holstein-Frisian cows had no significant effect on the relative mRNA abundance of *FASN* gene. However, the supplemented groups apparently had lower levels of expression of *FASN* gene than the control group (Figure 7.6). Week of supplementation and week by treatment interaction were inconsequential sources of variation (P>0.05; Table 7.4, Figure 7.6).

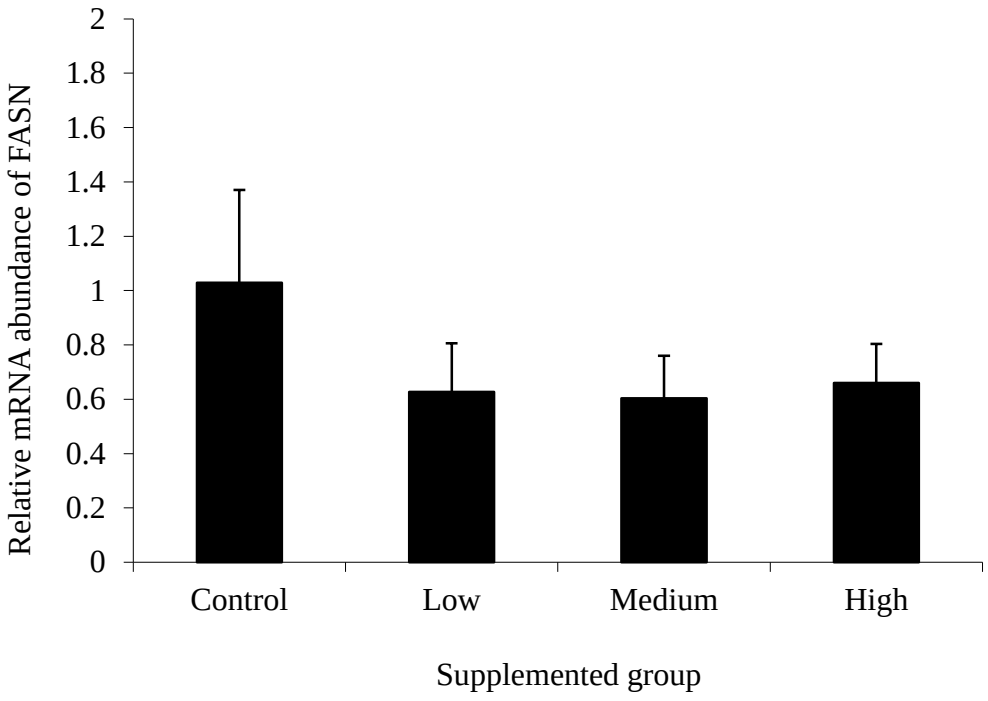

Figure 7.6 mRNA expression of the FASN gene in grazing cows supplemented with CDCO. Values are expressed as the geometric mean of the housekeeping genes/average Cp values for each gene. Values are means ± SEM.

Discussion

B-cell translocation gene 2 (BTG2)

The mammalian BTG2 gene belongs to the anti-proliferative (APRO) family of genes that regulate cell cycle progression in a variety of cell types (Roy *et al.,* 2005; Reiter *et al.,* 2014). BTG2 is a prototypical member of the BTG/TOB family with anti-proliferative properties. The protein encoded by this gene controls cell cycle progression and proneural gene expression by acting as a transcription co-regulator that enhances or inhibits the activity of transcription factors (Roy *et al.,* 2005; Reiter *et al.,* 2014). Thus, BTG2 has many functions involving regulation of cell growth, death, differentiation and survival (Mo *et al.,* 2011).

The present study found that supplementation of lactating Holstein-Friesian cows with CDCO repressed the expression of BTG2 significantly. The current result lends credence to the report of Jeckel et al. 2014 who demonstrated that in rats, BTG2 gene was down-regulated when dietary fatty acid diet was fed. Therefore, lack of BTG2 up-regulation in the in the blood of cows in the present study could be due to the fatty acid content of the fed diet. Although some of the BTG2 gene expression studies were on tumorous diseases in humans, significant expressions have been observed in pig muscle, uterus and heart where the gene appears to play a role in cell development (Feng *et al.,* 2007). A previous study also found that gonadotropin hormones stimulate the expression of BTG2 genes in the ovary during ovulation (Schmidt *et al.,* 2006). Usually, luteinising hormone surges during the pre-ovulatory period stops further growth of immature follicles, culminating in the ovulation of matured follicles for fertilisation (Li *et al.,* 2009; Park *et al.,* 2013). The BTG2 gene is thus an essential gene for normal reproduction in mammals. The greater number of services per conception observed in the CDCO supplemented group suggests that these cows ovulated later than the control cows (Figure 7.3). This statement is supported by the low expression of BTG2 in supplemented cows (Figure 7.5). It is well known that optimal recovery of the

cow's uterus following a normal gestation period requires growth, development and maturation of the granulosa/follicular cells for the cow to be ready for the next cycle of gestation. However, the anti-proliferative activity of the BTG2 gene might impact negatively on cell growth and tissue repair of the reproductive machinery. Our current findings seem to suggest that supplementation of cows with lipid-rich feeds could be utilised as a dietary manipulation tool to repress the expression of BTG2 gene and its anti-proliferative attributes.

Aralkylamine N-acetyltransferase (AANAT)

From published literature, the AANAT gene has been reported to be associated with long-chain omega-3 (LC-ω-3) polyunsaturated fatty acid (PUFA) synthesis (Perez *et al.,* 2010). A major cause for disparity between this report and our observation in the current study could be due to differences in dietary lipid sources, dosages fed and the relatively smaller proportion of LC-ω-3 PUFA in our experimental diet compared to the control diet (0.45% versus 13.30%; Table 7.2). The AANAT gene is also known to encode an acetyltransferase superfamily protein (Forrest *et al.,* 2003) that catalyses the rate-limiting step in the synthesis of melatonin from serotonin (Öner *et al.,* 2014) primarily found in the pineal gland (Piesiewicz *et al.,* 2012). Melatonin (N-acetyl-5-methoxytryptamine) is a hormone that plays a role in reproductive functions in mammals (Malpaux *et al.,* 1998), particularly in the growth and maturation of oocytes in the ovary and steroidogenesis in the granulosa cells via the mitogen-activated protein kinase pathway (Fiske *et al.,* 1984; El-Raey *et al.,* 2011). Melatonin is also essential for the function of the circadian clock that influences activity and sleep (Forrest *et al.,* 2003; Guo *et al.,* 2014). The mechanism by which melatonin regulates reproduction has been reported to be through the control of gonadotropin releasing hormone (GnRH) and gonadotropin inhibiting hormone (GnIH) receptors primarily found in the hypothalamic-pituitary axis to release gonadotropin hormones (Malpaux *et al.,* 1998; Soares *et al.,* 2003; Chowdhury et al., 2013). AANAT transcripts have been found to be

differentially expressed in high vs. low omega-3 index (O3I) muscles, suggesting a role for melatonin in reducing oxidative damage, including that of PUFA (Öner *et al.,* 2014). The ability of melatonin to protect against lipid peroxidation has been repeatedly documented in many studies using animal and plant tissues (Holman *et al.,* 2012). Spanish scientists reported that melatonin consumption assists in the control of weight gain since it stimulates the appearance of brown fat (beige), a type of fat cell that burns calories instead of storing them (Holman *et al.,* 2014). Their research demonstrated that melatonin treatment not only induced browning of inguinal white adipose tissue in Zucker diabetic fatty rats, but also increased thermogenic activity (Holman *et al.,* 2014). Taken together, these findings highlight the anti-obesity effect of melatonin and explain its metabolic benefits of protecting against oxidative degradation of PUFA in the muscle tissue thereby producing higher O3I levels (Holman *et al.,* 2014).

The afore-mentioned body of evidence in the published literature indicates that the expression of AANAT gene could play multifaceted functions in regulating fertility in dairy cows through biosynthesis of melatonin. This makes AANAT an ideal gene to explore in terms possible nutritional manipulation of its expression to assist in controlling seasonal breeding in pasture-based dairy systems. From the current study, the supplementation of Holstein-Friesian cows with CDCO was inconsequential to AANAT gene expression since no differential expression of the AANAT gene was observed between the treatment groups. This suggests that supplementing grazing primiparous cows with CDCO may not be an influential determinant of AANAT gene expression. However, further research studies are warranted with different dietary fat sources to establish ideal dosage levels that can strongly up-regulate/and or down-regulate AANAT in dairy cows before its use can be adopted by the industry.

Fatty acid synthase (FASN)

The lack of significant effect of the experimental lipid-rich diet on FASN expression is in agreement with the report of Bichi et al. (2013). It is pertinent to state that an observed trend in the current study towards the suppression of FASN expression (Figure 7.6) agrees with previous research in cattle (Thering *et al.,* 2009; Piesiewicz *et al.,* 2012; Qi *et al.,* 2014) and ewes (Hussein *et al.,* 2013). The observed suppression trend of FASN expression is consistent with the role of fats containing conjugated linoleic acid (CLA), especially trans-10, cis-12 18:2 (El-Raey *et al.,* 2011). The fatty acid profile of our treatment diet had a greater proportion of CLA than the control diet (1.48% versus 0.10%). Also the concentration of CLAa and CLAb in milk of cows consuming oil-supplement was greater than those for the control cows (Table 4.3). This could help to explain the down-regulation of FASN expression in cows supplemented with CDCO and also the suppression of the milk fat content (Figure 7.2). However, the discrepancy between the present and previous study (Harvatine *et al.,* 2009b) could be due to differences in the dietary fat source, dosage, level of polyunsaturated fatty acid intake and basal diet offered. Fatty acid synthase encodes a multifunctional enzyme that catalyses fatty acid synthesis (Strosberg, 1997). Fatty acid synthase is considered as a fundamental enzyme in *de novo* lipogenesis in mammals and its main function is to catalyse the synthesis of palmitate from acetyl-CoA and malonyl-CoA, in the presence of NADPH, into long-chain saturated fatty acids (LC-SFA; Strosberg, 1997; Wu et al., 2011). It has been shown that the FASN gene contributes to the regulation of body weight in humans, which results in the development of obesity (Strosberg, 1997; Wu et al., 2012). Thus, FASN is a complex homodimeric gene that plays a pivotal role in *de novo* lipid biosynthesis and regulation of milk fat content (Roy et al., 2006). One clear message from our results is the potential for supplementation of grazing cows with CDCO to down-regulate FASN expression. That way, the energy spared from reduced milk fat synthesis could be

partitioned towards milk production (Figure 7.1) particularly lactose (Palmquist and Jenkins, 1980) or metabolic functions in non-mammary tissues, especially, in reproductive tissues postpartum. This could prove highly significant to pasture-based dairy farmers in terms of managing the energy needs for production and reproduction in their herds. The downside to this assertion in pasture-based dairy systems is that milk fat is an economically important constituent of total milk solids upon which Tasmanian dairy farmers are paid in Australia. Furthermore, extreme suppression of FASN expression may be adverse to the butter manufacturing industries due to lower milk fat content. However, the beneficial impact is that the milk is being rid of mostly saturated fats, thus giving consumers a healthier product for which a premium can be charged to compensate for lower milk solids.

Conclusion

Dietary supplementation of grazing primiparous Holstein-Frisian dairy cows with CDCO had a significant influence in down-regulating the expression of BTG2. This might be important when the reproductive system of cows is recovering from the effect of gestation and new cell growth is required, but the downside is that hepatic gluconeogenesis could be hampered. mRNA abundance and expression of AANAT and FASN genes were not significantly impacted by CDCO supplementation in .spite of the observed trends towards up-regulation and down-regulation, respectively. The observed trend toward the suppression of FASN gene expression can be beneficial in sparing energy from milk fat synthesis and re-directing the surplus to non-mammary tissues *in vivo*. However, severe milk fat depression may be economically undesirable to Tasmanian dairy farmers because of its contribution to total milk solids upon which milk prices are based. These findings highlight the important role of nutrition in altering reproductive and lipogenic genes in the blood of lactating cows. Further studies with more experimental animals and CDCO supplementation levels would be required to confirm the current findings.

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Chapter 8 : Summary, Conclusions and Implications

High genetic merit cows have significantly large yield-driven energy demands that require high energy intake. Generally, pasture is insufficient to meet the energy requirements for postpartum lactation. Therefore, high-yielding dairy cows experience NEBAL at the beginning of lactation and a gradual but progressive decline in fertility.

In the first experiment, supplementation with CDCO independently influenced milk yield, fat, protein and lactose contents, whereas week/duration of supplementation affected both lactation and liveweight traits. These results indicate that the energy spared by CDCO-induced milk fat depression is partitioned toward increased milk yield and deposition as energy reserves in the adipose tissue to maintain good body condition in lactating cows. A cow in a good condition postpartum will have shorter postpartum anoestrous interval. However, severe milk fat and milk protein depression could have negative economic ramifications on dairy farm incomes because they are the primary constituents of butter and cheese production. However, the flip side of the coin could be a positive outcome in terms of an increase in the proportion of healthy LC-ω-3 PUFA in the milk of cows supplemented with CDCO.

The results presented in the second experiment demonstrated that as the level of supplementation with CDCO increased, the proportions of MUFA in the milk also increased at the expense of SFA, which is a clear indication of the nutritional modification of the milk towards healthier fat content. This implies that dairy farmers can utilise CDCO as a nutritional management tool to target niche markets willing to pay premium prices for healthy milk products. Furthermore, consuming milk, cheese and butter containing the same healthy nutrients as fish products, will ease the pressure on the finite wild fish species to

avoid potential extinction from Australian waters. Most importantly, these results indicate that consuming CDCO can increase the in vivo quantities of fatty acids reaching productive and reproductive tissues to manipulate lactation and fertility biomarkers.

Results of the third experiment on the impact of CDCO on plasma metabolites demonstrated that with the exception of BHB, CDCO at current levels of supplementation did not alter the plasma metabolite profiles of grazing primiparous cows. The lack of significant differences across treatments seems to indicate that higher levels of CDCO than the current levels used in this study, are probably needed. Furthermore, the duration of supplementation with CDCO had a greater impact on plasma metabolites than the levels of supplementation. These findings also indicate that primiparous cows grazing high quality pastures during spring had sufficient energy intakes to prevent negative energy balance at 40 days in milk without the need for added fat supplements. The effect of fat supplementation on plasma BHBA has been associated with the availability of carbohydrates and the impact of long chain fatty acids (>C18:0; particularly Docosahexaenoic acid, C22:6, C18:1, C18:3) on hepatic gluconeogenesis. As the level of CDCO supplementation increased, hepatic gluconeogenesis also increased due to low level of C22:6 and adequate levels of C18:1 and C18:3 in the treatment diet. The practical implication for dairy farmers in a pasture-based production system is that they don't need to spend money during spring on dietary energy supplements, but rather during winter or summer when pasture is scanty.

The early resumption of postpartum oestrus cycle is essential for reproductive performance in cows (De Fries *et al.*, 1998). However, it is dependent on the energy status and availability of adequate circulation of key reproductive hormones; P4, LH and FSH (Forde *et al*., 2011). The fourth experiment asked the following research question: "Will feeding incremental levels of CDCO to primiparous Holstein-Friesian dairy cows alter the concentrations of P4, FSH and LH *in vivo*?" It was clearly demonstrated that CDCO enhanced circulating plasma FSH

without affecting the concentrations of LH in plasma and P4 in milk under Australian pasture-based conditions. This will have practical beneficial implications for reproductive success in pasture-based dairy systems, considering that FSH is essential for growth, development and maturation of ovarian follicles. However, due to non-significant differences between supplemented and unsupplemented cows in P4 and LH, higher levels of CDCO than the current levels used in this study, are probably required. Furthermore, poor reproductive performance experienced by primiparous Holstein–Friesian cows grazing pasture might not be due to atypical hormonal profiles only, because other factors may be involved. The full extent of how lipid supplementation alters the dynamics of steroids and gonadotropic hormones in dairy cows still warranted further investigation into other molecular genetic factors such as gene expression and mRNA profiles of supplemented cows to provide a better understanding of CDCO's role in future applications as a dietary fat supplement for lactating cows.

Therefore, the fifth and final experiment was designed to investigate the effect of supplementation with incremental levels of CDCO on mRNA abundance and expression of genes encoding proteins required for optimal reproduction and *de novo* lipogenesis in pasture-based dairy cows. The results demonstrated that supplementation of cows with lipid-rich feeds could be utilised as a dietary manipulation tool to repress the expression of BTG2 gene and its anti-proliferative attributes which will help speed the postpartum recovery process of cows from the previous gestation period. The suppression of FASN expression indicates that milk fat is depressed, but the energy spared from reduced milk fat synthesis can be partitioned towards milk production and non-mammary tissues. This could prove highly significant to pasture-based dairy farmers in terms of managing the energy needs for production and reproduction in lactating herds. However, extreme suppression of FASN expression may be adverse to butter manufacturing industries due to lower fat content in milk.

Fortunately, it is mainly the saturated fats in the milk that are eliminated, thus giving consumers a healthier product containing more ω-3 and ω-6 PUFA, for which a premium can be charged to compensate for lower milk solids.

Taken together, the above findings are experimental evidence that CDCO is an essential fat-rich supplement that the Australian dairy industry can utilise to improve reproductive and fertility performance, whilst at the same time improving the quantity and quality of milk products without compromising health and well-being of the animals. Currently, the usage of canola oil supplements in the dairy industry is still scarce due to lack of information on its impact on production and reproduction performances of lactating dairy cows. Traditionally, Australian dairy farmers use silage, barley and wheat concentrates to supplement cows in times of scarce pasture production during the summer and winter seasons. Recently, resourcing these concentrates has become harder, mainly because of the increasing growth in the dairy industry driven by the demands from China and other Asian countries. Australia is also experiencing the harsh impacts of climate change, which is predicted to affect large scale production of barley and wheat in the future. Therefore, continuous use of these concentrates might prove very expensive in the long-run and canola oil might be considerably cheaper and more nutritious option for livestock feeds compared to the conventional wheat and barley concentrates currently required in large quantities to achieve profitable outcomes. Not only will canola oil be required in smaller quantities to achieve the same results, but the quantity and quality of the milk products will also improve where niche markets can be targeted for premiums. Furthermore, adequate concentrations of PUFA in canola oil imply that dairy cows feeding on this product will have improved fertility with better reproductive performance.

In summary, supplementing primiparous grazing dairy cows with CDCO:

- Increases milk yield, but at the expense of milk fat and milk protein;
- Has no negative impacts on body condition score and liveweight gain;
- In combination with high quality spring pasture prevents extreme NEBAL while the concentrations of NEFA and BHBA are low;
- Leads to healthier milk due to increased concentrations of MUFA and decreased SFA;
- Causes greater concentration of FSH *in vivo* without any effect on progesterone and luteinising hormones and;
- Alters the relative abundance and expression levels of fat-related genes involved in reproduction (BTG2 gene) and lipogenesis (FASN gene) *in vivo*.

Knowledge gaps that still warrant future studies are highlighted below:

- Assessment of costs associated with supplementing cows with CDCO requires an economic analysis to be conducted to determine the viability and profitability of producing milk with adequate concentrations of MUFA and less SFA.

- The finding in this thesis showed that CDCO induced milk fat depression. There is a need therefore to investigate the economic implication for extended milk fat depression on farm income and rural jobs.

- The timing and dosage of CDCO that can induce optimal production and reproduction benefits to dairy farmers.

- Impact of fat supplementation on cow immunity.

- Impact of processing on the milk content of SFAs, MUFAs and PUFAs.

- The type of cholesterol that affects P4 synthesis is not well known. Therefore, the need to profile the cholesterol (lipoproteins) obtained from lactating cows supplemented with CDCO is required.

- There is also the need for future studies with different dairy breeds under different management conditions.

- Ruminal biohydrogenation affects the chemical characteristics of supplemented fat, thus the post-ruminal contents might not contain the required levels of PUFAs. Therefore, it might be necessary to investigate the impact of encapsulated CDCO before feeding dairy cows.

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Appendices

This section contains all the information on published chapters, supplementary data and declarations.

Appendix 1

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BMC Veterinary Research

RESEARCH ARTICLE Open Access

Effect of crude degummed canola oil and *ad libitum* grazing on plasma metabolites of primiparous Holstein-Friesian cows in a pasture-based system

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Abstract

Background: The supplementation of fat to lactating dairy cows has long been used as a management tool to increase dietary energy density for improving cow production, reproduction and to alleviate negative energy balance. Attempts have been made to investigate the effect of canola meal on plasma metabolites in lactating cows, but the results have been diverse and inconsistent. To our current knowledge, there is a dearth of published information on the utilization of Crude Degummed Canola Oil (CDCO) in pasture-based dairy systems. Therefore, the objective of this study was to investigate the changes in plasma metabolite profiles of pasture-based, primiparous, Holstein-Friesian cows supplemented with varying dietary levels of CDCO for eight weeks. The study tested the hypothesis that *feeding grazing primiparous Holstein-Friesian cows for eight weeks with incremental levels of CDCO supplement will decrease plasma non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA), but increase plasma cholesterol and glucose metabolites.*

Results: Twenty lactating primiparous Holstein-Friesian cows 40 days in milk were randomly allotted into four treatment groups that consisted of a wheat-based, pelleted basal diet with no supplemental CDCO (control), basal diet with CDCO added at 25 ml/kgDM (DM; dry matter) (low), 35 ml/kgDM (medium) and 50 ml/kgDM (high) in an eight-week feeding trial, after two weeks of adjustment. Treatment influenced BHBA but had no effect on plasma NEFA, cholesterol and glucose metabolite profiles ($P > 0.05$). However, week of supplementation had a significant effect ($P < 0.05$) on BHBA, NEFA and glucose concentrations.

Conclusions: We concluded that with the exception of BHBA, CDCO at current levels of supplementation does not alter the plasma metabolite profiles of grazing primiparous cows. The lack of significant differences across treatments seems to indicate that higher levels of CDCO than the current levels used in this study, are probably needed. Furthermore, the duration of supplementation with CDCO had a greater impact on plasma metabolites than the levels of supplementation. Our findings also suggest that primiparous cows grazing high quality pastures during spring have sufficient energy intakes to prevent negative energy balance at 40 days in milk without the need for added fat supplements.

Keywords: Primiparous Holstein-Friesians, Crude degummed canola oil, Supplement, Plasma metabolites

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Appendix 2

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Effect of incremental levels of crude degummed canola oil on milk progesterone, plasma luteinizing and follicle stimulating hormones of primiparous Holstein–Friesian cows in a pasture-based system

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Luteinizing hormone;
Follicle stimulating hormone

Abstract Dietary supplementation of lactating cows with fat can alter the profiles of key reproductive hormones and boost postpartum energy balance. However, published data under Australian pasture-based dairy production conditions are scanty and inconsistent. Therefore, the objective of this study was to determine whether dietary inclusion of crude degummed canola oil (CDCO) at incremental levels for eight-weeks will have significant influence on progesterone (P4), luteinizing hormone (LH) and follicle stimulating hormone (FSH) of primiparous Holstein–Friesian cows grazing pastures. We tested the hypothesis that postpartum supplementation of primiparous Holstein–Friesian cows with dietary CDCO in a pasture-based system will alter the concentrations of P4, LH and FSH reproductive hormones. A random allocation of twenty primiparous Holstein–Friesian cows into four treatment groups that consisted of a wheat-based pelleted basal diet with no supplemental CDCO (control), or a wheat-based pelleted basal diet with CDCO added at 25 ml/kg (low),

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Peer review under responsibility of Faculty of Veterinary Medicine, Cairo University.

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Appendix 3

Journal of Nutritional Therapeutics, 2014, 3, 55-66 55

Influence of Supplementing Pasture-Based Primiparous Holstein-Friesian Dairy Cows with Crude Degummed Canola Oil on Milk Fatty Acid Composition

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Abstract: The quest for alternative sources of healthy nutrients that facilitate the modification of milk without compromising drinking quality is a continuous research endeavour. The objective of the study was to quantify the milk fatty acid composition of pasture-based primiparous Holstein-Friesian dairy cows supplemented with crude degummed canola oil (CDCO) with a view to improving the milk quality for beneficial health effects. This study tested the hypothesis that incremental supplementation of grazing primiparous Holstein-Friesian cows with CDCO will alter milk fatty acid composition towards increased total monounsaturates. Comparisons were made between unsupplemented grazing dairy cows and their peers on dietary supplements containing low (25ml/Kg DM), medium (35ml/Kg DM) or high levels (50ml/kg DM) of CDCO in addition to *ad libitum* grazing access to pasture. There was no significant effect ($p>0.05$) of CDCO supplementation for eight weeks on the proportions of total polyunsaturated fatty acids (*f*PUFA), omega-3 (ω-3) and omega-6 (ω-6) fatty acids in milk. However, significant impacts of CDCO were observed on the proportions of 18:1ω9c, 18:1ω7t, total saturated (*f*SFA) and total monounsaturated (*f*MUFA) fatty acids ($p<0.005$), with a significant increase in the *f*MUFA/*f*SFA ratio in cows consuming CDCO. It was concluded that incremental levels of CDCO supplementation can modify the fatty acid composition of milk towards increased monounsaturates without any negative impact on grazing primiparous cows.

Keywords: Monounsaturated Fatty Acids, Polyunsaturated Fatty Acids, Saturated Fatty Acids, omega-3, omega-6.

INTRODUCTION

The demand for milk and other dairy products has slightly increased in Australia, with the consumption of drinking milk per capita rising from 104.4 liters in 2010/11 to 106.2 liters in 2011/12, respectively [1]. The primary focus of dairy farmers is to increase milk production with adequate fat and protein compositions because of the associated economic benefits of milk solids. In response to health concerns about coronary heart disease, obesity and arteriosclerosis, research interests in modifying milk fatty acid composition toward less saturated medium-chain (≤C12) fatty acids and more long-chain (≥C18) polyunsaturated fatty acids (PUFA) are on the increase. The simplest way of altering milk fat composition is to supplement the diets of cows with unsaturated lipids [2,3]. Milk fat composition is changed more by the amount and composition of dietary fat than any other dietary component, and several studies [4-6] have been published on the response of milk fat composition to dietary lipid supplements in dairy cows. However, in Tasmania's pasture-based dairy production system, dietary supplementation of lactating cows with fat is not a common nutritional management practice, mainly because of its unknown impacts on milk fatty acid composition and other lactation traits. Previous fat studies in other dairy systems have reported the effects of fat supplements on milk fatty acid profiles [2, 3, 6]. Dietary fat supplementation of dairy cows in pasture-based production systems has been targeted toward enhancing the proportions of ω-3 and ω-6 fatty acids at the expense of SFA to achieve desirable human health benefits [7-9]. However, the beneficial health effect of fat supplements can be countered by the concurrent production of *trans*-monounsaturated fatty acids (MUFA) known to be associated with cholesterol [10, 11]. However, published studies in Australia

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Appendix 4

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Reproduction and Fertility Parameters of Dairy Cows Supplemented with Omega-3 Fatty Acid-rich Canola Oil

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Authors' contributions

This work was carried out in collaboration between all authors. Author JRO researched and wrote the first draft of the manuscript as part of his PhD thesis literature review. Authors MJF, BSMA, PAL, PDN and AEOMA contributed in the design, reading and making needed changes to the final manuscript as a review article. All authors read and approved the final manuscript.

Review Article

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Published 7th February 2014

ABSTRACT

Dietary supplementation of lactating dairy cows with fat has long been used as a management tool to increase the energy density of feeds, improving milk production, reproduction and alleviating the menace of postpartum negative energy balance. In this paper, we show that while attempts have been made to investigate the effects of omega-3 (n-3) polyunsaturated fatty acid (PUFA) on reproduction and fertility parameters the results have been diverse and inconsistent. The effect of n-3 fatty acids on prostaglandin F2α

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Appendix 5

Additional Materials

The tables and figures presented here are supplementary. The materials contain data that fail to contribute further information that can support the acceptance or rejection of the predetermine hypotheses. Some of these data could not be added to the chapters because of publication limitations.

Chapter 3: lactation and liveweight traits

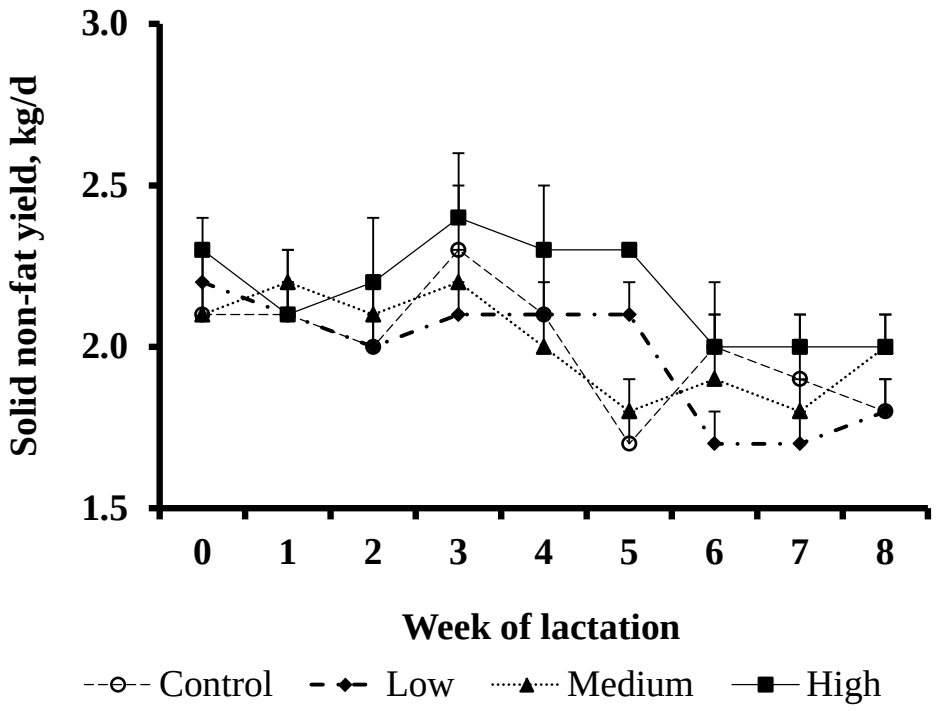

Figure 1 Weekly variation in milk solid non-fat percentage (a) and milk solid non-fat yield (b) in primiparous Holstein-Friesian cows in control, low, medium and high levels of canola oil supplementation.

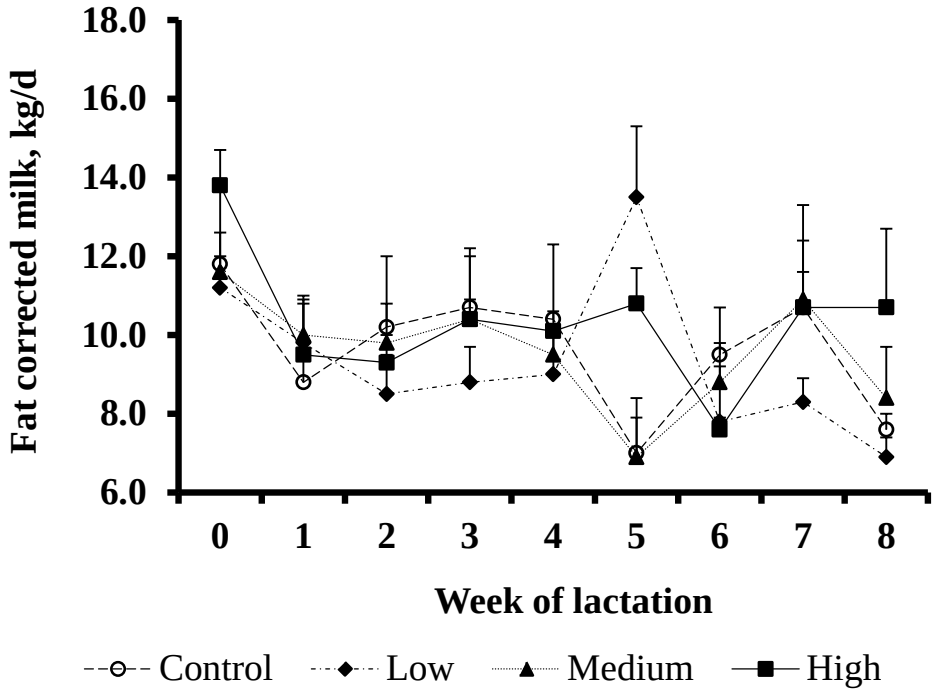

Figure 2 Fat corrected milk in primiparous Holstein-Friesian cows in control, low, medium and high levels of crude degummed canola oil supplementation.

Chapter 4: Milk fatty acid profiles

Table 1 Mean fatty acid (±SE) content (mg/100g) of milk samples from primiparous Holstein-Friesian cows fed on control, low, medium and high levels of canola oil supplementation over five weeks.

Fatty acid	Treatment group				P-value	Week of supplementation						RMSE	P-value	
	Control	Low	Medium	High	TRT	0	2	4	6	7	8		WK	TRT*WK
14:0	629.74±61.3	606.54±37.0	520.81±51.4	570.88±48.2	NS	520.6±36.9	520.33±43.0	551.66±62.7	735±85.8	594.84±64.3	569.54±57..5	273.84	NS	NS
15:0	91.91±14.4a	60.74±4.3b	54.09±4.8b	55.15±4.5b	**	49.85±2.8	64.40±4.2	77.29±19.0	78.2±12.6	66.23±8.3	56.89±6.5	44.29	NS	NS
16:1	15.65±1.7a	14.83±1.1ab	11.35±1.0b	11.99±1.0b	*	10.23±0.8	12.42±0.9	14.55±2.2	15.42±1.9	14.99±1.6	13.16±1.3	6.73	NS	NS
16:0	1509.3±154.4	1256.67±92.5	1070.3±111.0	1179.56±100.4	NS	1124.82±82.3	1052.13±94.4	1155.54±136.3	1505.66±206.1	1388.87±151.5	1296.72±161.4	640.74	NS	NS
17:0	31.1±3.7a	22.43±1.4b	22.07±1.8b	22.86±1.4b	*	24.25±1.1	22.32±1.3	26±4.5	27.86±3.9	25.32±2.5	21.94±2.1	12.03	NS	NS
18:3w6	1.10±0.1	1.09±0.1	0.92±0.1	0.87±0.1	NS	1.01±0.1	0.87±0.1	0.91±0.1	1.29±0.2	1.02±0.1	0.89±0.1	0.62	NS	NS
18:4w3	0.95±0.2b	1.21±0.3ab	1.51±0.3ab	2.06±0.4a	*	0.81±0.3	1.71±0.4	1.42±0.5	1.22±0.4	1.78±0.4	1.65±0.3	1.61	NS	NS
18:2w6	95.58±11.4	87.56±8.7	85.86±8.5	103.03±8.8	NS	104.82±6.4a	93.19±7.2a	113.23±15.0a	102.9±14.2a	86.05±10.5ab	57.87±10.0b	48.91	*	NS
18:3w3	40.77±6.3	37.21±5.1	38.41±5.0	43.92±5.1	NS	44.97±2.6a	45.23±4.5a	47.97±7.3a	49.8±8.4a	36.11±6.4a	16.40±5.7b	27.16	**	NS
18:1w9c	710.43±67.76	660.9±47.6	722.06±57.4	797.55±48.6	NS	816.6±46.0	594.71±48.4	664.11±63.4	775.91±89.3	777.86±77.1	707.23±71.6	294.60	NS	NS
18:0	328.91±39.4a	312.72±31.3a	356.3±34.2a	364.01±28.4a	**	492.95±32.7	289.35±29.1	272.33±35.2	361.64±52.6	323.65±37.7	303.02±37.2	165.63	NS	NS
18:2CLAa	50.01±5.5	46.79±4.2	46.54±4.6	56.51±3.8	NS	43.15±2.2bc	46.05±4.7abc	52.04±5.3abc	61.47±7.7a	57.62±6.4ab	39.47±4.5c	23.06	*	NS
18:2CLAb	10.18±0.9	9.30±0.8	9.24±0.6	11.51±0.7	NS	9.42±0.6	9.71±0.7	10.40±1.2	9.88±1.3	10.08±0.8	10.88±1.0	4.14	NS	NS
19:0	2.25±0.3	1.92±0.2	2.17±0.2	2.43±0.2	NS	2.59±0.1	2.02±0.2	2.15±0.4	2.42±0.4	2.02±0.3	1.98±0.3	1.29	NS	NS
20:5w3	3.88±0.3	3.63±0.3	3.26±0.3	3.25±0.3	NS	4.07±0.3	2.77±0.3	3.57±0.4	3.91±0.5	3.36±0.3	3.37±0.3	1.64	NS	NS
20:3w6	3.48±0.4	3.27±0.2	2.69±0.3	2.82±0.2	NS	3.19±0.3	2.42±0.2	3.02±0.4	3.52±0.4	3.24±0.4	3.02±0.3	1.54	NS	NS
20:4w3	1.67±0.2	1.62±0.2	1.54±0.2	1.65±0.2	NS	2.26±0.2a	1.38±0.2b	1.61±0.2b	1.66±0.3b	1.53±0.2b	1.29±0.2b	0.88	*	NS
20:2w6	1.15±0.2b	1.66±0.3b	1.45±0.2b	3.36±0.4a	***	2.40±0.4	1.94±0.4	2.40±0.5	1.62±0.3	1.46±0.3	1.62±0.3	1.52	NS	NS
20:0	4.09±0.5	4.24±0.5	4.56±0.5	5.58±0.5	NS	5.83±0.4	3.95±0.5	4.23±0.7	5.14±0.8	4.13±0.5	4.44±0.6	2.65	NS	NS
22:6w3	0.39±0.1	0.29±0.1	0.28±0.1	0.21±0.1	NS	0.43±0.1	0.15±0.1	0.38±0.1	0.39±0.1	0.19±0.1	0.22±0.1	0.42	NS	NS
22:4w6	0.30±0.1	0.40±0.1	0.20±0.1	0.16±0.1	NS	0.06±0.0b	0.09±0.1b	0.19±0.1ab	0.45±0.1a	0.41±0.1a	0.40±0.1a	0.38	**	NS
22:5w3	5.81±0.6	5.58±0.4	4.84±0.5	4.85±0.4	NS	5.05±0.4	4.06±0.4	5.15±0.6	6.53±0.9	5.34±0.5	5.52±0.5	2.60	NS	NS
22:0	2.16±0.2	1.85±0.2	2.03±0.2	2.08±0.1	NS	2.25±0.1	1.84±0.1	1.80±0.2	2.12±0.3	1.88±0.2	2.29±0.2	0.81	NS	NS
24:0	0.90±0.2	0.53±0.1	0.52±0.1	0.55±0.1	NS	0.87±0.1	0.51±0.1	0.40±0.2	0.80±0.2	0.45±0.2	0.73±0.2	0.71	NS	NS
∑SUM	4424.85±421.5	3990.28±236.4	3756.58±318.4	4191.09±280.0	NS	4130.12±234.4	3649.71±284.1	3939.47±436.0	4713.56±546.1	4275.83±411.2	3835.53±367.8	1754.11	NS	NS
∑SFA	2896.57±296.1296.0	2550.64±155.7	2261.51±209.2	2451.21±193.5	NS	2479.24±165.8	2191.68±188.1	2369.78±291.5	3047.25±375.7	2663.07±283.2	2488.89±258.8	1205.42	NS	NS
∑MUFA	1308.35±115.4	1235.59±84.6	1294.7±100.9	1502.01±84.0	NS	1425.51±71.0	1245.36±97.8	1323.34±136.3	1416.85±157.6	1400.31±125.4	1199.62±115.5	517.48	NS	NS
∑PUFA	219.93±24.5	204.05±19.1	200.37±19.4	237.87±18.7	NS	225.38±12.4a	212.67±19.2ab	246.36±30.3a	249.47±34.1a	212.45±23.8ab	147.02±21.6b	106.96	*	NS
∑ω-3 PUFA	53.49±7.3	49.53±5.8	49.83±5.8	55.94±5.7	NS	57.58±3.4a	55.29±5.1a	60.09±8.7a	63.49±10.0a	48.3±7.3ab	28.45±6.5b	31.73	*	NS
∑ω-6 PUFA	109.96±12.7	101.24±9.6	97.65±9.5	117.01±9.8	NS	118.38±7.3a	104.75±8.1ab	127.42±16.7a	117.84±16.1a	99.59±11.6ab	70.83±11.2b	54.90	*	NS
∑ω-3LC-PUFA	11.76±1.1	11.11±0.8	9.92±1.0	9.97±0.9	NS	11.81±0.8	8.35±0.9	10.7±1.3	12.48±1.7	10.41±1.0	10.4±1.0	5.20	NS	NS

[1]∑SFA is the sum of 12:0, 13:0, i14:0,14:0, i15:0, a15:0,15:0, i16:0, 16:0, i17:0, 17:0, i18:0, 18:0, 19:0, 20:0, 20:0, 22:0, 24:0; ∑MUFA is the sum of 14:1ω-5c, 15:1ω-6c, 16:1ω-9c, 16:1ω-7c, 16:1ω-7t, 16:1ω-5c, 16:1,17:1ω-8+a17:0, 17:1ω-6c, 18:1ω-9c, 18:1ω-7c, 18:1ω-7t, 18:1ω-5c, 18:1a, 18:1b , 20:1ω-11c, 20:1ω-9c, 20:1ω-7c, 20:1ω-5c, 22:1ω-11c, 22:1ω-9c, 22:1ω-7c, 24:1ω-11c, 24:1ω-9c, 24:1ω-7c; ∑PUFA is the sum of 18:3ω-6, 18:4ω-3, 18:2ω-6, 18:3ω-3, 18:2CLAa, 18:2CLAb, 20:4ω-6, 20:5ω-3, 20:3ω-6, 20:4ω-3, 20:2ω-6, 22:5ω-6, 22:6ω-3, 22:4ω-6, 22:5ω-3; ∑ω-3 LC-PUFA is the sum of 20:5ω-3, 20:4ω-3, 22:6ω-3, 22:5ω-3; ∑ω-3 PUFA is the sum of 18:4ω-3, 18:3ω-3, 20:4ω-3, 20:5ω-3, 22:6ω-3, 22:5ω-3; ∑ω-6 is the sum of 15:1ω-6, 17:1ω-6, 18:2ω-6, 18:3ω-6, 20:4ω-6, 20:3ω-6, 20:2ω-6, 22:5ω-6, 22:4ω-6.

[2]TRT=treatment feed, WK= Week

[3]RMSE = root mean square error.

[4] NS = no significance; * = significant (P<0.05); ** = highly significant (P<0.01); *** = very highly significant (P<0.001). These descibe the supplement feeds, weeks and their interaction.

[5]Means with different superscript (a,b, c) are significantly different.

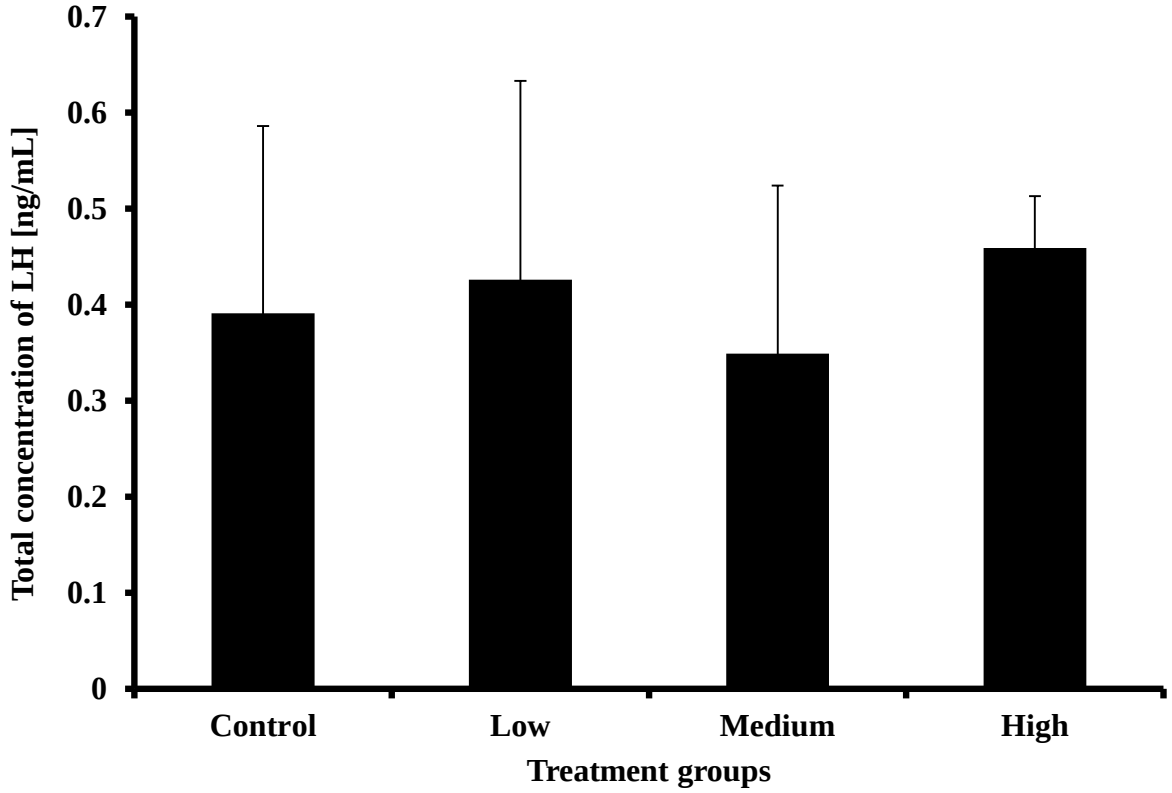

Figure 1 Total mean concentrations of LH of primiparous Holstein-Friesian cows receiving 0ml kg^{-1}DM (control), 25ml kg^{-1}DM (low), 35ml kg^{-1}DM (medium) and 50ml kg^{-1}DM (High) levels of CDCO treatments for eight weeks. Each group had five cows.

Appendix 6

Declarations

This section provides the co-authorship declarations, describing J.R. Otto's active involvement with chapters and manuscript preparation according to the University of Tasmania, School of Land and Food PhD thesis by publication guidelines. Publication status of chapters is described explicitly in the List of publication and appendices 1-4.

Article title:

Reproduction and fertility parameters of dairy cows supplemented with omega-3 fatty acid-rich canola Oil

Co-authors:

J.R. Otto, M.J. Freeman, B.S. Malau-Aduli, P.D. Nichols, P.A. Lane and A.E.O. Malau-Aduli

Evaluation scale:

1 – has contributed to this work (10-33%)

2 – has made substantial contribution to this work (34-66%)

3 – has made a major contribution to this work (67-100%)

Declaration regarding specific elements	***Extent (1,2,3)***
1. Formulation/identification of the scientific problem that need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments	
2. Planning of the experiments and methodology design, including selection of methods and method development	
3. Involvement in the experimental work	
4. Presentation, interpretation and discussion in a journal format of the obtained data	
Overall contribution	

Explanation of student involvement in the work:

Active involvement in: research, design and analysis of the literature, and composition of the review paper.

Signature of the co-authors:

Article title:

Effect of dietary supplementation of pasture-based primiparous Holstein-Friesian cows with degummed crude canola oil on body condition score, liveweight, milk yield and composition

Co-authors:

J.R. Otto, P. Nish, R. Balogun, M. Freeman, B.S. Malau-Aduli, P.A. Lane and A.E.O. Malau-Aduli

Evaluation scale:

1 – has contributed to this work (10-33%)

2 – has made substantial contribution to this work (34-66%)

3 – has made a major contribution to this work (67-100%)

Declaration regarding specific elements	***Extent (1,2,3)***
1. Formulation/identification of the scientific problem that need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments	
2. Planning of the experiments and methodology design, including selection of methods and method development	
3. Involvement in the experimental work	
4. Presentation, interpretation and discussion in a journal format of the obtained data	
Overall contribution	

Explanation of student involvement in the work:

Involvement in; experimental/methodology design, Planning/logistics of the field work, sample and data collection, laboratory and statistic data analysis and composition of paper.

Signature of the co-authors:

Article title:

Influence of supplementing pasture-based primiparous Holstein-Friesian dairy cows with crude degummed canola oil on milk fatty acid composition

Co-authors:

J.R. Otto, B.S. Malau-Aduli, P.D. Nichols and A.E.O. Malau-Aduli

Evaluation scale:

1 – has contributed to this work (10-33%)

2 – has made substantial contribution to this work (34-66%)

3 – has made a major contribution to this work (67-100%)

Declaration regarding specific elements	***Extent (1,2,3)***
1. Formulation/identification of the scientific problem that need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments	
2. Planning of the experiments and methodology design, including selection of methods and method development	
3. Involvement in the experimental work	
4. Presentation, interpretation and discussion in a journal format of the obtained data	
Overall contribution	

Explanation of student involvement in the work:

Involvement in; experimental/methodology design, Planning/logistics of the field work, sample and data collection, laboratory and statistic data analysis and composition of paper.

Signature of the co-authors:

Article title:

Effect of crude degummed canola oil and *ad libitum* grazing on plasma metabolites of primiparous Holstein-Friesian cows in a pasture-based system

Co-authors:

J.R. Otto, B.S. Malau-Aduli, R.O. Balogun, P. Nish and A.E.O. Malau-Aduli

Evaluation scale:

1 – has contributed to this work (10-33%)

2 – has made substantial contribution to this work (34-66%)

3 – has made a major contribution to this work (67-100%)

Declaration regarding specific elements	***Extent (1,2,3)***
1. Formulation/identification of the scientific problem that need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments	
2. Planning of the experiments and methodology design, including selection of methods and method development	
3. Involvement in the experimental work	
4. Presentation, interpretation and discussion in a journal format of the obtained data	
Overall contribution	

Explanation of student involvement in the work:

Involvement in; experimental/methodology design, Planning/logistics of the field work, sample and data collection, laboratory and statistic data analysis and composition of paper.

Signature of the co-authors:

Article title:

Effect of incremental levels of crude degummed canola oil on milk progesterone, plasma luteinizing and follicle stimulating hormones of primiparous Holstein- Friesian cows in a pasture-based system

Co-authors:

J.R. Otto, B.S. Malau Aduli, A. Rao, I.J. Clarke and A.E.O. Malau Aduli

Evaluation scale:

1 – has contributed to this work (10-33%)

2 – has made substantial contribution to this work (34-66%)

3 – has made a major contribution to this work (67-100%)

Declaration regarding specific elements	***Extent (1,2,3)***
1. Formulation/identification of the scientific problem that need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments	
2. Planning of the experiments and methodology design, including selection of methods and method development	
3. Involvement in the experimental work	
4. Presentation, interpretation and discussion in a journal format of the obtained data	
Overall contribution	

Explanation of student involvement in the work:

Involvement in; experimental/methodology design, Planning/logistics of the field work, sample and data collection, laboratory and statistic data analysis and composition of paper.

Signature of the co-authors:

Article title:

Effect of supplementation with crude degummed canola oil on the expression of fat-related genes involved in reproduction and lipogenesis in primiparous Holstein-Friesian dairy cows in a pasture-based system

Co-authors:

J. R. Otto, B. Suybeng, A. Kashani, P.A. Lane, B.S. Malau-Aduli, P.D. Nichols and A.E.O. Malau-Aduli

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